Production of
di-D-Fructofuranose 1,2´:2,3´ Dianhydride (DFA III)
using Recombinant Inulase II

Von der Gemeinsamen Naturwissenschaftlichen Fakultät
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zu Braunschweig

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Dissertation

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To my mother,
Anca Letca

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Summary

Chapter 1

Introduction

In the last years, applied microbiology has become a major growth area since the recombinant DNA technology offers the possibility to take advantage of the cellular synthetic capacities. Whereas up till then random mutation and selection had been used to increase the level of preexisting activity in a microbial cell, recombinant DNA technology is now being used to confer to the cells an entirely new synthetic capacity, such as, for instance, the synthesis of human hormones by *Escherichia coli*. The recent advances in molecular biology provide the possibility to perform industrial large-scale bioprocesses targeted on the production of high-value added products. For instance, the production of human growth hormone could be recently scaled up to 200 l bioreactors, while the traditional products of microorganisms (amino acids and antibiotics) are frequently produced in 200-400 m^3 reactor scales *(Sahm, 1999)*.

Also the enzyme employed in this work, inulase II, was recently produced as recombinant protein in genetically modified *E. coli* (see 1.4.). For this, the gene for inulase II was transferred from the natural producer, a strain of *Arthrobacter* spec., to *Escherichia coli (Jahnz, 2001)* and fermentation experiments were performed up to 20 l bioreactor scale *(Jahnz, 2001; present work)*. The inulase II was further used to obtain DFA III from inulin as a row material.

A considerable number of processes using immobilized biocatalysts for converting sugars and polysaccharides into new products, oligosaccharides and derivatives, mostly sweeteners and functional food, is applied industrially, several are under development. They comprise hydrolytic as well as synthetic reactions by hydrolyses and glycosyltransferases.

Oligosaccharides of the inulin type are commonly known as fructooligosaccharides (FOS) and exhibit prebiotic qualities. Commercially, fructooligosaccharides may be derived by enzymatic treatment of sucrose or by greater or lesser degree of hydrolysis or fractionation of inulin derived from Jerulasalem artichoke or chicory. Yacon also represent a potential commercial source of FOS for which minimal processing is required *(Douglas et al., 2002)*. Oligosaccharides in food and agriculture are currently garnering much attention, especially in their application as prebiotics (non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and the activity of one or a limited number of bacterial species in the colon) *(Gibson and Roberfriod, 1995)*. A range of oligosaccharides is produced by glycosyltransferases including both glucosyl- and fructosyltransferases and using sucrose or starch, or dextrins, respectively, as convenient, high quality and low price commercial substrates. Oligosaccharides currently produced for commercial markets include cyclomaltrodextrins, maltodextrins, fructooligosaccharides, galactooligosaccharides, soy oligosaccharides, etc. Isomaltooligosaccharies have found interest in the field of food, pharmaceutical and cosmetics due to their utility for prevention and treatment of diseases in immune or other biological functions, microbial infections and tumors *(Buchholz and Seibel, 2002)*.

Cyclodextrins are products of major importance in the fields of food, pharmaceuticals (drug protection, slow release) and commodities used in textile drying (for odor removal and as a perfume carrier). In food their function may be aroma complexation and slow release, stabilization of flavors, reducing

bitterness etc. They are furthermore used to remove cholesterol from milk and egg products. They form inclusion complexes with a wide variety of hydrophobic guest molecules leading to the applications mentioned and can be obtained via enzymatic reaction using cyclodextrin glycosyltransferases from starch or dextrins are *(Buchholz and Kasche, 1997).*

1.1. The Aim of the Project

While Arthrobacter strains secrete inulase II in the medium, *E. coli* accumulates the protein intracellularly in high amounts. A fermentation leading to high amount of biomass (so-called high cell density fermentation, HCDF) will therefore lead to a significant amount of activity in a fermentation experiment.

The aim of this work was to acquire high volume activity of inulase II by performing HCDF of recombinant *E. coli* strains (*E.coli/*pMS*ift*OptWT and *E.coli/*pMS*ift*OptR) which could serve for the optimized production of DFA III *(Walter, 2001)*. For this, different fed-batch strategies and fermentation media employed in the technical scale were tested and evaluated in terms of inulase II activity.

The recombinant enzymes were characterized and related to the already existing information available for inulase II isolated from other bacterial strains. Furthermore, the recombinant inulase II enzyme solutions were assayed for immobilization experiments using anion-exchangers resins, followed by a comparison of immobilized versus unbound enzyme under kinetic aspects.

1.2. Inulin

The substrate for inulase II is inulin. Inulin is widely distributed in nature in more than 3,000 plant species and a few microorganisms. It is located in vacuoles and functions as plant storage carbohydrate. Among the many inulin-containing plant species (Jerusalem artichoke, chicory, dahlia, leek, onion, garlic, etc) the Jerusalem artichoke and chicory are generally considered as a major new agricultural crop *(Fuchs and Voragen, 1988).*

Inulin is a linear β-2, 1-linked fructose polymer terminated by a sucrose residue. Its chemical structure is presented in figure 1.1.

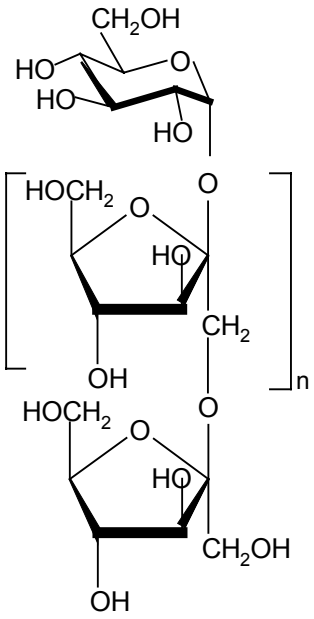


Figure 1.1. The chemical structure of inulin (6< n < ~60)

The polymerization degree (DP) is generally less than 30, though in some plants it is up to 200 and in fungi up to 100,000. The plant species, the maturity of the plant and the time and storage methods are determinant factors for the degree of

inulin polymerization. The chain length ranges from 6 to 60 units (*VanHaastrecht, 1995)*. Dahlia inulin has a polymerization degree around 20, which is rather high as compared to Jerusalem artichoke inulin (DP 6) or chicory inulin (DP 10-14) *(Leenheer, 1996)*.

As a structural characteristic, inulin is mostly linear but could be also branched in some species.

The inulin molecule can be transformed either chemically, enzymatically or microbially. Many transformations proceed without previous hydrolysis to fructose, with only partial hydrolysis (when fructo-oligosaccharides are produced) or with initial hydrolysis to fructose *(Fuchs, 1989)*.

Most of the inulin commercially available presently as industrial food ingredient is extracted from chicory roots. The root of *Cichorium intybus* contains ~ 15-20% inulin *(Niness, 1999)*, which can be extracted with hot water. After extraction, the inulin can be hydrolyzed chemically or enzymatically to FOS or fructose. Since inulin is not digested in the upper gastrointestinal tract, inulin has a reduced caloric value and does not lead to a rise of serum glucose or stimulate insulin secretion. In addition, it stimulates the growth of intestinal bifidobacteria *(Roberfroid et al., 1998).*

1.3. Inulase II

An enzyme which converts inulin into di-D-fructofuranose 1,2´:2,3´ dianhydride (DFA III) was found and isolated from the culture supernatant of *Arthrobacter ureafaciens* in 1972 by Tanaka *(Tanaka et al., 1972)*. The enzyme was named inulin fructotransferase (depolymerizing) and classified as E.C. 2.4.1.93. In the CAZY data bank, the enzyme is classified in the family 91 (www. afmb.cnrs-mrs.fr/CAZY) *(Reilly, personal communication)*. Up to now, enzymes with inulin fructotransferase function (inulinase II) have been identified and isolated from a

large spectrum of microorganisms, mostly belonging to the *Arthrobacter* genus and some *Pseudomonas* species. Some characteristics of these enzymes are listed in table 6.3.

The enzyme attacks (2→1)-β-linked fructan molecules from the non-reducing fructose ends and requires the presence of at least two adjacent (2→1)-β-fructo-furanosyl linkages. Referring to inulase II isolated from *Arthrobacter globiformis* C11-1, one unit of enzyme is defined as the amount of enzyme, which produces 1.0 µmol of DFA III per minute at 30°C and pH 5.0 *(Haraguchi, 1988).*

After the complete digestion of inulin, the reaction products are found to be DFA III (60%), and FOS (40%) (GF_2, GF_3, GF_4) (see below). Since inulase II cannot use GF_2, GF_3 and GF_4 as substrates, 1-D-fructofuranosyl-GF_4 seems to be the smallest substrate for *Arthrobacter* sp. H65-7 inulase II *(Yokota, 1991, 1)*. On the other hand, the inulase II isolated from *Arthrobacter ureafaciens* has been reported to degrade also GF_3 and GF_4 into DFA III *(Uchiyama, 1975).*

- DFA III - di-D-fructofuranose 1,2´:2,3´ dianhydride
- FOS - fructo-oligosaccharides
- GF_2 - kestose
- GF_3 - nystose
- GF_4 – fructosyl-nystose

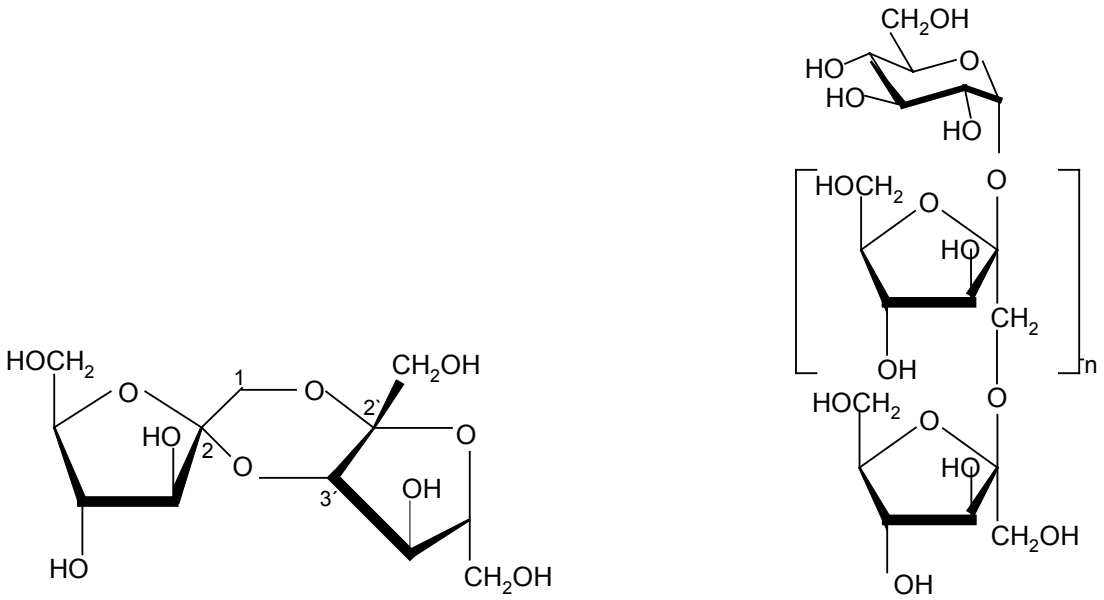


Figure 1.2. Chemical structure of (a) DFA III and (b) fructo-oligosaccharides (n=2 - kestose; n=3 – nystose; n=4 – fructosyl-nystose)

- <u>di-D-fructofuranose 1,2´:2,3´ dianhydride (DFA III)</u>

The dianhydride has one of the anomeric centers in the α-configuration and the other (C-2´) in β-configuration, so according to the nomenclature rules the complete name for DFA III will be α-D-fructofuranose β-D- fructofuranose 2´,1:2,3´- dianhydride. During the inulin conversion into DFA III, the anomeric configurations of the product is not conserved from the substrate in the enzymatic tranglycosylation reaction, but are created anew *(Uchyiama, 1982)*. DFA III is a non-reducing sugar, having a melting point of 162°C and an optical rotation $[\alpha]_D^{20}$ of 135,6° *(Neubauer, 1998)*.

DFA III is expected to be utilized in various applications due to its properties:

- it has half of the sweetness of sucrose
- it is low caloric since it is not metabolized in the gastro intestinal tract of mammals
- it is chemically more stable than fructo-oligosaccharides due to the presence of a dioxane ring
- it has growth promoting activity for such enteric bacteria as *Bifidobacteria (Teeuwen et al., 1992)*

1.4. Strains Employed in this Work

For the production of bacterial proteins, procaryotic microorganisms are often employed as hosts in recombinant DNA technologies, for instance the gram-negative *Escherichia coli* is used to obtain unglycosylated proteins since the posttranslational modification of recombinant protein is not possible for these bacterial cells. Not only that it is a well-characterized biological system, *Escherichia coli* cultures develop with high growth rates and their development offers the possibility to establish and perform robust cultivation techniques. Many plasmids and expression vectors are presently commercially available for the synthesis of heterologous proteins using *Escherichia coli*.

Recombinant DNA methods were employed in order to develop a biological system capable to express an enzyme with inulase II activity, used for the conversion of inulin to DFA III *(Walter, 2001)*. For this, a gene coding for inulase was expressed in *Escherichia coli* as a host strain.

Inulase II is naturally produced by different species of the genus *Arthrobacter,* e.g. *A. ureafaciens* 7116 *(Uchiyama, 1973)*, *A. globiformis* C 11-1 *(Haraguchi,*

1988), *A. aurescens* IFO 12136 and *A. ilicis* MCI-2297 and of the genus *Pseudomonas*, for example by *Pseudomonas fluorescens* No. 949 *(Uchiyama et al. 1989, 1)*. Also the isolate from *Arthobacter* genus identified as *Arthrobacter* sp. Bu0141 is able to produce extracellular inulase II. The enzyme isolated from this strain is thermostable at high temperatures (up to 70°C), a determinant fact for the industrial production of DFA III. *Arthobacter* sp. Bu0141 DNA was used to isolate the inulase II gene employed in this work for recombinant inulase production. The obtaining of the recombinant *E. coli* strains expressing inulase II has been already described before *(Walter 2001)* and some characteristics will be mentioned below.

The *ift*-gene coding for inulase II was isolated from *Arthrobacter* sp. Bu0141, cloned into a λ-phage and subcloned in *E.coli*. Following, the whole gene was isolated in two Bam HI fragments. The genetic map of these fragments (MS*ift*BH2 and MS*ift*BH1) is presented in figure 1.3 (taken from Walter, 2001).

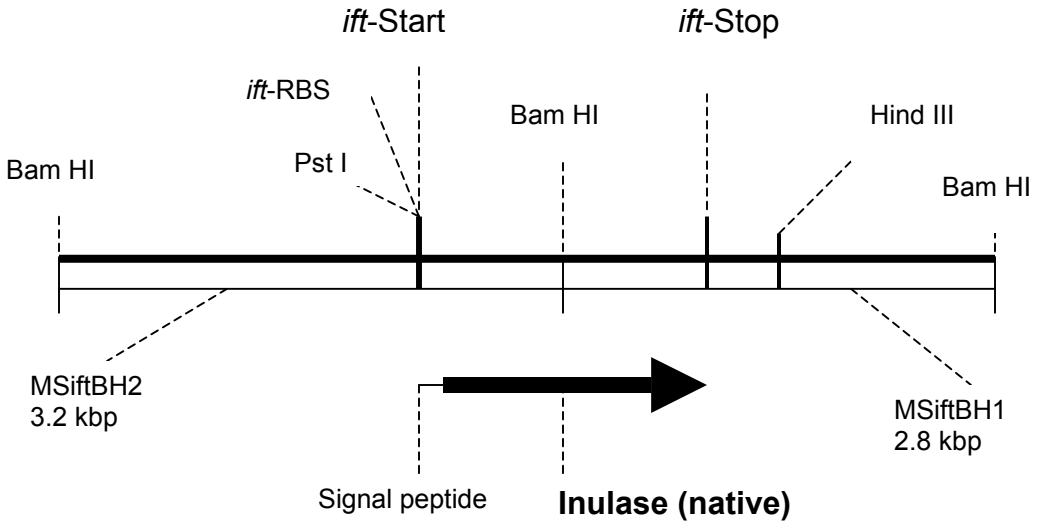


Figure 1.3. Genetic map of Bam HI fragments MS*ift*BH2 and MS*ift*BH1. A part from MSiftBH2 (3.2 kbp) encodes for the N-terminus half and MSiftBH1 (2.8 kbp) for the C-terminus half of the *ift*-gene. Both Bam HI fragments were isolated from the total genomic DNA from *Arthrobacter* sp. Bu0141. Pst I and Hind III – singular restriction sites used for the construction of expression matrix, *ift*-RBS – ribosome-binding site, *ift*-Start and *ift*-Stop – enclose the coded region. The translation product (450 amino acids) of *ift* gene (1,350 nucleotide) is the native inulase (410 amino acids) preceded by a signal peptide (40 amino acids). The arrow indicates the translation direction.

The fragment MS*ift*BH2 (3.2 kbp) codes for the N-terminal half and the MS*ift*BH1 (2.8 kpb) for the C-terminal half of the *ift*-gene. Both fragments were isolated from the total genomic DNA from *Arthrobacter* sp. Bu0141. Preceding the sequence coding for inulase II is a short piece which was assumed to be a signal peptide. The function of this signal peptide is with high probability the posttranslational export of the protein out of the cell. The signal peptide is no longer present in the mature and catalytically active subunit of inulase II, so it is possibly cleaved off during or after the transport across the membrane *(Schubert, personal communication).* It seems to be responsible for RNA stability and for the folding during translation. Obviously this signal peptide cannot be recognized in

E. coli, for the expression of the wild-type gene of *Arthrobacter* spec. Bu0141 did not lead to an excretion from the *E. coli* cells. Since a cleaving off after translation is also highly improbable, the DNA sequence for the signal peptide was deleted, leading to an enzyme activity increase. This shortened DNA sequence was called Ms*ift*OptWT and was used to modify *E. coli* for the expression of inulase II enzyme. For this purpose, the cloning vectors pUC 18 and pUC 19 were employed.

An expression construct pMS*ift*OptWT was obtained introducing the matrix MS*ift*OptWT in the pUC 19 plasmid. The Ms*ift*OptWT fragment was placed between the cleavage sites for the restriction endonucleases Hind III and Eco RI downstream of Lac-ribosome binding-site. The gene map of the pMS*ift*OptWT matrix is represented in figure 1.4 (after *Walter, 2001*). With this sequence cloned into a high copy vector (pUC 19), an inulase II activity of about 320,000 U/l was obtained after cell disruption. The expression product contains 418 amino acids and the enzyme with inulase II activity begins at amino acid position 9.

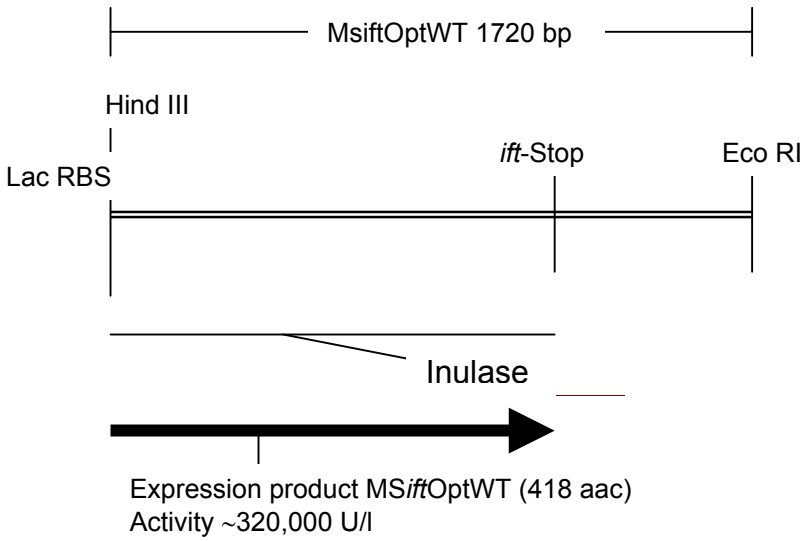


Figure 1.4. The genetic map of pMS*ift*OptWT cloning vector

With this efficient matrix, an error-prone polymerase chain reaction assay was performed; different clones were tested for the inulase II activity and a mutant with 35 % higher activity was isolated (Walter, 2001). A closer examination revealed a replacement of a nucleotide at position 661, where guanine (G) was exchanged for adenine (A). At the amino acid level, this results in arginine (R) instead of glycine (G) at position 221. The corresponding expression matrix was named pMS*ift*OptR, it lead to an inulase II activity of about 435, 000 U/l.

Further the plasmids obtained as described above were introduced into the strain *E. coli* XL-1 Blue (Stratagene Corp., La Jolla, USA) and the recombinant *E .coli* strains *E. coli*/pMS*ift*OptWT (DSMZ* 13463) and *E. coli*/pMS*ift*OptR (DSMZ 13465) were investigated in the frame of this work.

* Deutschen Stammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig)

Chapter 2

Theoretical Background

2.1. High Cell Density Fermentation

A bacterial cultivation can be performed in batch, fed-batch and continuous fermentation processes *(Yee and Blanch, 1992)*. These three variants are characterized by the following parameters:

- batch processes – all nutrients are initially present in the bioreactor; only O_2, CO_2 and H_2 as well as titrants for the pH control and antifoaming agents may be supplied or removed during the cultivation, and the product is removed at the end of experiment.
- fed-batch processes – the initial amount of nutrients is used to initiate the cultivation. In a first phase, the culture develops in batch conditions at a maximum specific growth rate. In the second phase (fed-batch phase), nutrients are added into the bioreactor at desired feed rates to control the concentration of nutrients and consequently the cellular growth rate. The product is removed at the completion of the process.
- continuous processes – the nutrients are continuously supplied and the product is withdrawn from the bioreactor at appropriate flowing rates to maintain a constant fermentation volume.

Generally, continuous culture is considered to provide the highest productivity (the amount of product obtained per unit time) among the cultivation techniques in the case of a non-degenerative microorganism *(Mori, 1983)*. The continuous cultivation is not very suitable though for genetically modified microorganisms, since a loss of plasmid or the structural changes in the expression vectors have to be avoided *(Ryan and Parulekar, 1991, Hellmuth et al., 1994).* Various factors have to be considered in optimizing recombinant protein productivity. Besides the parameters related to the host organisms, e.g. the host physiology, the site of recombinant product accumulation, the molecular strategy for maximizing gene expression, the cultivation conditions have to meet also the strain growth and production necessities *(Kleman and Strohl, 1994)*. These specific needs can be best fulfilled under fed-batch conditions, which are often employed to obtain high cell density cultures. Generally, recombinant protein production is proportional to the biomass concentration and in order to increase both cell density and productivity the cultivation conditions have to be optimized. In this attempt, a strong influence over process development is exercised by the adopted feeding strategy. Constant or exponential feeding profiles are designed to encounter the strains particularities, being optimized to provide critical nutrients for growth, to prevent product degradation or to minimize the formation of toxic products.

High cell density cultivations (HCDC) techniques developed for recombinant *Escherichia coli* fermentations have been successfully carried out adopting different fed-batch strategies. These techniques had to overcome important drawbacks as substrate or nutrient inhibition, limited oxygen transfer capacity, the formation of growth-inhibitory by-products or limited heat dissipation *(Rinas, 1998)*. Some nutrients could inhibit *E. coli* cell growth above certain concentration limits: glucose (> 50 g/L), ammonia (>3 g/L), iron (> 1.15 g/L), magnesium (> 8.7 g/L), phosphorous (> 10 g/L) and zinc (> 0.038 g/L) *(Riesenberg, 1991, 1).* Therefore, HCDCs are initiated with concentrations below the inhibitory thresholds, and nutrients are added as necessary to achieve the fermentation aim.

Inhibitory concentrations of metabolic by-products are reached during the high-cell cultivation processes. For *E.coli* cultivations, acetate is the main inhibitory by-product, and is produced when:

- the carbon flux into the central metabolic pathway exceeds the biosynthetic demands and the capacity for energy generation within the cell (C-source excess) *(Holms, 1986)*
- oxygen becomes a growth-limiting factor
- high partial pressure of CO_2 is reached *(Pan et al. 1987).*

Acetate has been reported to have a greater detrimental effect on recombinant cells than on non-recombinant cells *(Koh et al., 1992)* affecting both the cell growth and the recombinant protein expression *(Bech-Jensen and Carlsen, 1990; Brown et al., 1995).* Even though the exact mechanism of this effect has not been elucidated it seems that acetate represses the synthesis of DNA, RNA, proteins and lipids. The inhibitory effect of acetate is intensified by the salts that accumulate as a consequence of pH control *(Lee, 1996)*. Furthermore it was observed that acetate inhibition is more accentuated in complex media than in defined media. Taking into consideration these negative effects the fermentation strategies aiming to attain high cellular densities and high productivity are designed to minimize acetate production. The feeding is controlled to maintain C-source growth-limiting conditions and in addition to prevent the dissolved oxygen concentration of becoming a limiting factor.
As a result of mass transfer limitations in high-density cultivation, oxygen can rapidly become a limiting growing-parameter. Since oxygen consumption increases with the growth rate, reducing the growth rate leads to a lowered need for oxygen.

Some fed-batch strategies for recombinant *E.coli* cultivations have been performed at constant feeding rate when the feeding solution insures C-limiting conditions and the nutrients amount relative to the biomass concentration will decrease as the culture develops. As a result, the specific growth rate (μ) will

decrease over time. The characteristics of constantly fed-batch culture is the linear increase of biomass during the fermentation process. Using this technique for *E. coli* MC 1061 expressing recombinant human growth hormone precursors (x-hGH), a dry cell weight of 30 g/L was obtained. *(Bech-Jensen and Carlsen, 1990*).

An often successful strategy appears to be the exponential feeding rate. As a result, a constant concentration of a growth-limiting substrate can be maintained in the cultivation bioreactor. The culture develops at a constant specific growth rate, low enough to minimize inhibitory by-product accumulation ($\mu < \mu_{max}$). For *E. coli* fermentations, acetate formation was detected when the specific growth rate exceeds a certain critical growth rate. Therefore the feeding rate is appropriately chosen to determine a lower growth rate, less than half of the maximum specific growth rate *(Riesenberg and Guthke, 1999)*. The feeding rate ($F_{\exp}$) that allows exponential growth with a constant specific growth rate can be calculated as follows:

$$F_{\exp} = F_0 \times \exp(\mu^S (t - t_0)) \tag{2.1}$$

where: $F_{\exp}$ = the feeding flow rate during cultivation (L/h)

t = the fermentation time (h)

t_0 = the fermentation time when the feeding procedure starts (h)

F_0 = the feeding flow rate at the beginning of fed-batch phase (L/h)

μ^S = the preset growth rate (h^{-1})

with F_0 calculated as:

$$F_0 = \frac{\mu^S \times x_{batch} \times y_{S/x}}{S_0} \times V_f \qquad (2.2)$$

where: μ^S = the preset growth rate (h^{-1})

x_{batch} = the optical density at the end of batch cultivation

$y_{S/x}$ = the substrate yield (g/L)

V_f = the fermentation volume (L)

S_0 = concentration of substrate in feeding solution (g/L)

Using C-limiting conditions for *E. coli* TG1 strain cultivation, cell concentrations up to 148 g(DCW)L^{-1} have been achieved for glycerol-limiting exponential feed in a stirred tank reactor *(Korz et al., 1995)*.

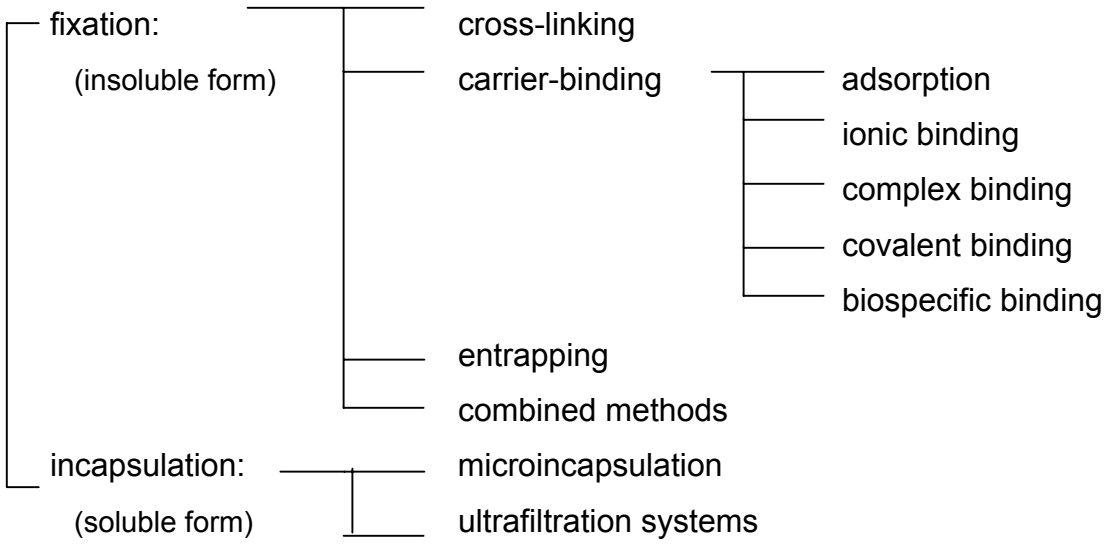
The highest cell densities reported to date are 190 g(DCW)L^{-1} for *E.coli* K12 *(Märkl et al., 1997)* in a dialysis reactor and 175.4 g(DCW)L^{-1} for a recombinant *E.coli* XL-1 blue strain producing poly(3-hydroxybutyrate) *(Lee, 1994).*

2.2. Immobilization on Anion-exchangers

Enzymes are large protein molecules with chemically reactive groups, ionic groups, and hydrophilic as well as hydrophobic domains that can all participate in the immobilization process through physical adsorption, ionic binding, or covalent linkage.
The biocatalysts are immobilized mainly to allow repeated or continuous use of their activity in a controlled environment. Adsorption of an enzyme molecule to a carrier can also stabilize its tertiary structure.

The immobilized enzyme is defined as “the enzyme physically confined or localized in a certain defined region of space with retention of its catalytic activity, which can be used repeatedly and continuously” *(Chibata, 1987).*

The immobilization methods can be classified as follows *(Buchholz and Kasche, 1997)*:



In figure 2.1 some immobilization techniques are represented.

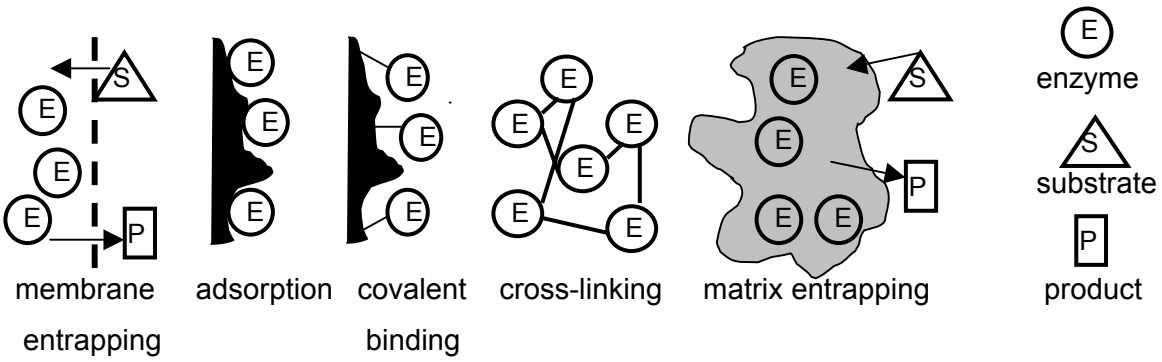


Figure 2.1. Schematic representation of some immobilization methods

An universal immobilization method does not exist. Each end use requires evaluation of the individual steps according to criteria such as the purpose of immobilization, activity, yield, stability, simplicity and economic feasibility. Each method has its advantages and disadvantages that have to be considered for every biocatalyst and enzymatic reaction investigated.

The adsorption of an enzyme onto an insoluble support is the simplest method of enzyme immobilization. The major components of an immobilized enzyme system are the support (carrier), the enzyme and the mode of interaction of the enzyme with the support.

The carrier

The support may be a membrane, a water-insoluble solid, or a polymer matrix. The support (carrier) should have the following properties *(Scouten, 1987)*:

- large surface area and high permeability
- sufficient functional groups for enzyme attachment under non-denaturing conditions
- hydrophilic character or hydrophobic, depending on the specific case
- water insolubility

- chemical and thermal stability
- mechanical strength
- resistance to microbial attack
- regenerability
- toxicologically safe
- low price

Carriers can be classified according to their morphology (porous, nonporous, or gel type) and their chemical composition. The most widely used supports are listed in table 2.1.

Table 2.1. Chemical classification of carriers used for enzyme immobilization (from *Kennedy and Cabral,1987)*.

Organic carriers	**Inorganic carriers**
• natural polymers	• minerals
- polysaccharides	- attapulgite clays
- cellulose	- bentonite
- starch	- kieselghur
- dextran	- pumice stone
- agar and agarose	
- alginate	
- carrageenan	
- chitin and chitosan	
- proteins	
- gelatin	
- silk	
- carbon materials (activated C)	

• synthetic materials - polystyrene - polyacrylates and polymethacrylates - polyacrylamide - glycidyl methacrylates - maleic anhydride copolymers - polyamides	• synthetic materials - nonporous glass - controlled pore glass and metal oxides - metals

The selection of the carrier is based on the compatibility of the matrix with the surface, for example the polarity of the biocatalyst´s active form, taking into consideration the isoelectric point of the protein (the pH at which the net charge is zero, meaning that the number of positive and negative charges is equal). Proteins will have either a net negative charge or net positive charge depending upon the isoelectric point of the protein and the pH of the solution and thus it is possible to use anion or cation exchanger immobilization. The anion-exchanger immobilization is carried out at pH values above the isoelectric point of the protein. The immobilization process will have to consider the interaction of the catalyst with the resin surface, mass transfer phenomena in the catalytic reaction, mechanism of catalysis, the influence of the bonding of catalysts to carriers, the influence of microenvironment and the inactivation and stabilization mechanisms *(Buchholz, 1987)*. Some relevant parameters affecting the performances of the immobilized biocatalysts are summarized in table 2.2.

Table 2.2. Parameters affecting the performances of the immobilized biocatalysts (from Buchholz, 1987)

	Physical and chemical parameters involved
Maximum activity or initial reaction rates	T, P, *t*, pH, buffer conc.
Effectiveness η as a function of	
External mass transfer	d_p, *v, u* or d_i, *n*
Pore diffusion	d_p, v_{max}, K_M, T, S, P
Operational stability, depending on abrasion, enzyme inactivation, irreversible. adsorption, occlusion, etc	*t*, S, P, T, pH, conc. of other components

The parameters summarized above are determinant for the selection of an appropriate immobilization technique and carrier selection. Types and concentration of carrier functional groups are essential characteristics with respect to enzyme immobilization. They determine the coupling reaction and the physico-chemical properties of the immobilized biocatalyst. Functional groups most often involved are carboxyl and amino groups. The anion-exchangers investigated in the frame of this paper are weakly basic resins with amino functional groups (see table 5.1).

The interaction of the enzyme with the support

Attachment of the enzyme to the carrier surface is governed by physical interactions such as van der Waals forces, hydrogen bonding, or hydrophilic-hydrophobic effects *(Woodward, 1984)*. Although the biocatalyst may be

immobilized without conformational changes associated with loss of activity, the binding is sensitive to environmental conditions, such as ionic strength, pH and temperature. A subsequent cross-linking step helps to stabilize the immobilized biocatalyst on the carrier. Bi- or multifunctional reagents such as glutaraldehyde, toluene diisocyanate or bisdiazobenzidine derivatives can be used as cross-linking agents.

The yield of immobilized enzyme is used to characterize the immobilization efficiency and is defined as the ratio of immobilized protein based on enzyme offered for binding *(Buchholz, 1979)*.

The operational stability of an immobilized biocatalyst is quantified by the catalytic half-life ($\tau_{1/2}$), defined as the period of time in which the catalytic activity of the enzyme decreases to 50% of its initial value. The catalytic activity will decline as a function of time under operational conditions. Due to the complex nature of the decay mechanism and mass transfer limitations, which could mask the decay function, extrapolation methods for the catalytic half-life determination, have to be avoided in order to obtain reliable information.

2.3. Kinetics Parameters

The Michaelis-Menten model accounts for the kinetic parameters of many enzymes *(Howaldt and Chmiel, 1991)*. This model may be applied to the enzymatic biotransformation of inulin using inulase II which can be regarded as a first order reaction in early stages *(Neubauer, 1998; Jahnz, 2001).*

The mechanism for a reaction catalyzed by a soluble enzyme in many cases assumes the reversible formation of an enzyme - substrate (ES) complex, which decomposes irreversibly into product (P) and free enzyme (E):

$$S + E \leftrightarrow ES \longrightarrow E + P$$

The maximal reaction rate and the Michaelis-Menten constant can be obtained from the general equation:

$$v = v_{\max} \frac{[S]}{[S] + K_M} \tag{2.3}$$

where: $v_{\max}$ = maximal reaction rate

$[S]$ = substrate concentration

K_M = Michaelis-Menten constant

The reaction rate furthermore is a function of the substrate and enzyme concentrations, pH, temperature, solvent composition, and ionic strength.

From equation 2.3, particular cases can be differentiated:

- when $[S] << K_M \Rightarrow$ $v = \frac{[S] \times v_{\max}}{K_M}$ (2.4)

  (the reaction rate is directly proportional to the substrate concentration)

- when $[S]_{>>} K_M \Rightarrow v = v_{\max}[S]$ (2.5)

  (the reaction rate is maximal, independent of substrate concentration)

- when $[S] = K_M \Rightarrow v = \frac{v_{\max}}{2}$ (2.6)

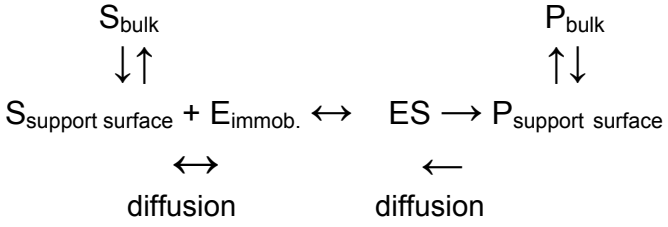
  (the K_M is equal to the substrate concentration at which the reaction rate its half of its maximum value)

$$\frac{[S]}{v} = \frac{K_M}{v_{\max}} + \frac{1}{v_{\max}} \times [S]$$

Plotting $\frac{[S]}{v}$ versus $[S]$ (Hanes-Wolf linearization) one can obtain a straight line with an intercept $\frac{K_M}{v_{\max}}$ and a slope $\frac{1}{v_{\max}}$.

The reaction mechanism for an <u>immobilized</u> enzyme is more complex because:

1. the enzyme activity may be changed by immobilization
2. the substrate must be transported to the support and the product must be transported from the reaction site back into the bulk solution:



The reaction rate is governed by additional parameters resulting from immobilization as conformational changes, chemical changes, enzyme – support interactions and diffusion effects.

In the frame of this work, the same approximations were utilized for immobilized enzymes as for the soluble enzymes.

In addition, the kinetic parameters for both soluble and immobilized enzyme were also determined using the Runge-Kutta integration procedure. This method is supported by a computer program developed by Demuth *(Demuth, 1994)*, which minimizes the influence of the calculation errors over the values of the kinetic parameter.

Chapter 3

Cell Disruption

3.1. Introduction

Inulase II is an extracellular protein secreted by *Arthrobacter* sp. When produced in recombinant *Escherichia coli/*pMS*ift*OptR and pMS*ift*OptWT the signal peptide responsible for the posttranslational export of the protein out of the cell is missing *(Walter et al., 2001)*. The sequence of *Arthrobacter* would not have been recognized by *E.coli* anyway. The Inulase II remains in the cell and in order to recover it from the cytoplasm it is necessary to disrupt the cells.

The type of energy input necessary to isolate the proteins that are accumulated within the cells permits the cell disruption classification in:

- mechanical cell disruption processes and
- nonmechanical cell disruption processes, including chemical, biological and physical methods.

Each method has specific advantages and disadvantages, depending on the product and its applications. Mechanical disintegration is generally applicable, while the other approaches need specific procedures for each individual case. Disruption of a small volume of cell suspension in a laboratory for analytical purposes is generally performed using mechanical forces and especially

important are the stability of the cell wall and the size of the cell itself. For instance, animal tissue can be damaged by very small shear forces but the Gram-positive bacteria require a larger energy input *(Atkinson and Mavituna, 1991; Schwedes and Bunge, 1990)*.

The release of Inulase II from *E. coli* cells was performed using mechanical disruption methods: for small volumes, the mixer mill and the ultrasounds device were used, and for bigger volumes a high-pressure homogeniser (GBF laboratory).

3.2. Mixer mill

Cells are disrupted by shear forces generated during vibration and enforced motion of glass beads in the mixer mill *(Hummel and Kula, 1989)*. The protein release for the disruption in the mill is a first-order rate process. The most important parameters to influence the efficiency of cell disruption are: the size of the beads, cell concentration, ratio of cell suspension to the amount of glass beads and the total volume of suspension and beads *(Schütte and Kula, 1988)*.

To establish the optimal conditions for the cell disruption using the mixer mill, experiments were performed using a non-modified *E.coli XL-1 blue* strain. The cells from an overnight LB medium culture were harvested and resuspended in phosphate buffer + Mg^{2+}. The magnesium ions are cofactors for the Benzonase, the enzyme used to reduce the viscosity of the suspension due to nucleic acids. For every g of cell suspension 0.2 µl Benzonase (Merck, Darmstadt) were used. The necessity of Benzonase is a critical step for the use of this method for large-scale disruption considering the price of the enzyme.
The plasmid that contains the gene for Inulase II is not present in *E.coli XL-1 blue* cells and so the disruption evaluation was made by quantifying the total protein

concentration. It was assumed that cell breakage would be the same for the non-modified *E.coli*.

3.2.1. Ratio of cell suspension to glass beads

Experiments were performed varying the cell concentration of the tested suspension. Different amounts of beads (0.3 mm diameter) were added to a constant volume of cell suspension to find an optimum ratio of cell suspension and beads. The size of the beads was chosen according to the studies of Schütte and Kula *(Schütte and Kula, 1993)*, which found that for bacteria suspension the appropriate bead size varies in the range of 0.2-0.5 mm and for yeasts in the range of 0.4-0.7 mm. The disintegration was carried out at room temperature with precooled samples. Considering that Inulase II is stable up to 60°C, the temperature increase during disruption had no influence on the enzyme activity. The highest temperature recorded after proceeding the disruption for 30 minutes was 29°C.

As control parameter for the cell disruption efficiency, the protein concentration was determined according to Bradford *(Bradford, 1976)*.

The ratio glass beads: cell suspension was varied from 0.5:1 to 3:1 and all samples were treated for 30 minutes at maximum amplitude of the mixer-mill. The results presented in table 3.1 were obtained for a 5% cell suspension and a 40% degree of loading.

Table 3.1. Influence of the ratio of beads: cell suspension ratio on protein release

Ratio Beads: cell suspension	Protein concentration (µg/ml)
0.5:1	1,500
1:1	2,400
1.5:1	3,000
2:1	3,800
3:1	3,850

The degree of cell disruption increases with the amount of glass beads as demonstrated by the release of protein.

For further experiments a ratio of 2:1 of glass beads and cell suspension was considered as appropriate. Since there is no significant difference between the results obtained for 2:1 ratio and 3:1 ratio, the use of a higher amount of beads is not necessary.

3.2.2. Cell suspension concentration

Suspensions containing different cell concentrations were tested and the results are presented in figure 3.1. The cells were suspended in phosphate buffer, so that the final suspension concentration was varying in the range of 2 to 30% wet cell weight (WCW).

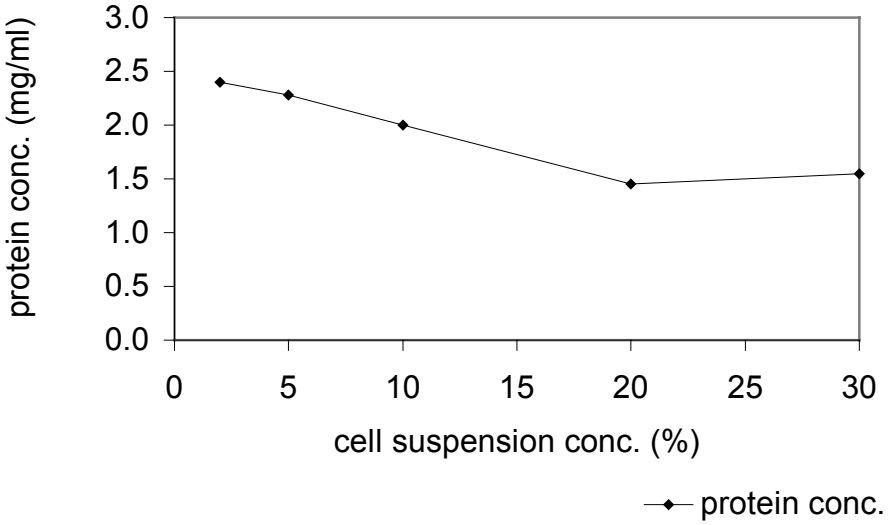


Figure 3.1. Release of protein during disruption for different cell concentrations

The cell disruptions were performed under identical conditions for all samples (ratio beads: suspension- 2:1, 40 % loading degree and 30 minutes). The highest protein concentration was obtained for 2% cell concentration, which means that the protein release using grinding with glass beads is less efficient with the increase of the cell suspension concentration as is known from the literature. For further experiments involving the mixer-mill cell disruption a suspension concentration of 2% WCW was employed.

3.2.3. Bead Loading

Different degrees of filling the 30 ml disruption vials with beads were tested. Using a 2 % cell suspension, a ratio of 1:2 for the suspension and beads and a disruption time of 30 minutes, the vial loading degree was varied in a range of 30 to 90%. The results are shown in figure 3.2.

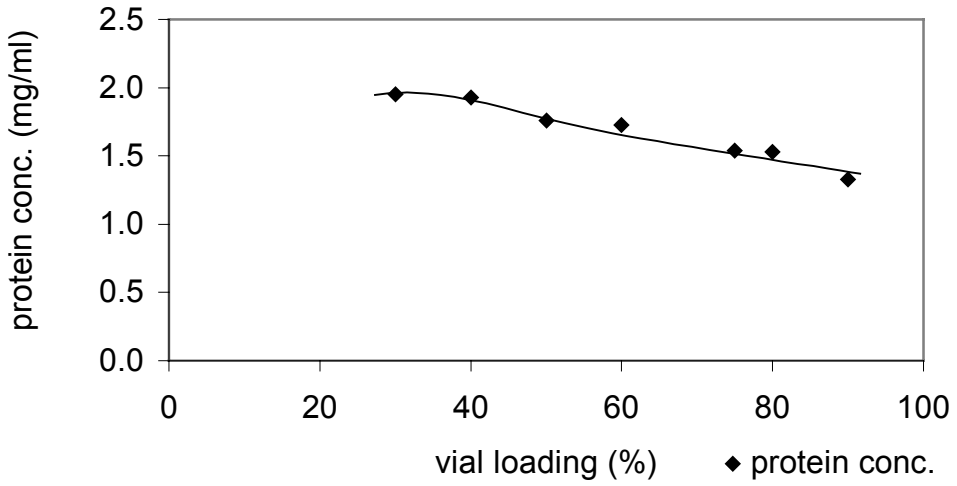


Figure 3.2. Effect of vial loading on the protein release.

The loading volume influenced the efficiency of disruption as shown in figure 3.2. The protein concentrations obtained are decreasing with the loading volume at a degree of loading higher than 40%, and so for a 90% degree of loading, the protein concentration was 1.3 times lower than for 30%. Therefore, a loading degree of 40% was considered as appropriate for further experiments.

3.2.4. Disruption Time

The disruption time is as well a very important parameter for the efficiency of protein release. The protein concentration was measured after 25, 30, 35 and 40 minutes for two different concentrations of cell suspension (2 and 5%), 40% loading degree and 2:1 ratio of glass beads: suspension. For a better comparison of the 2% and 5% suspensions, the protein concentrations were calculated with reference to a 1% cell suspension (figure 3.2).

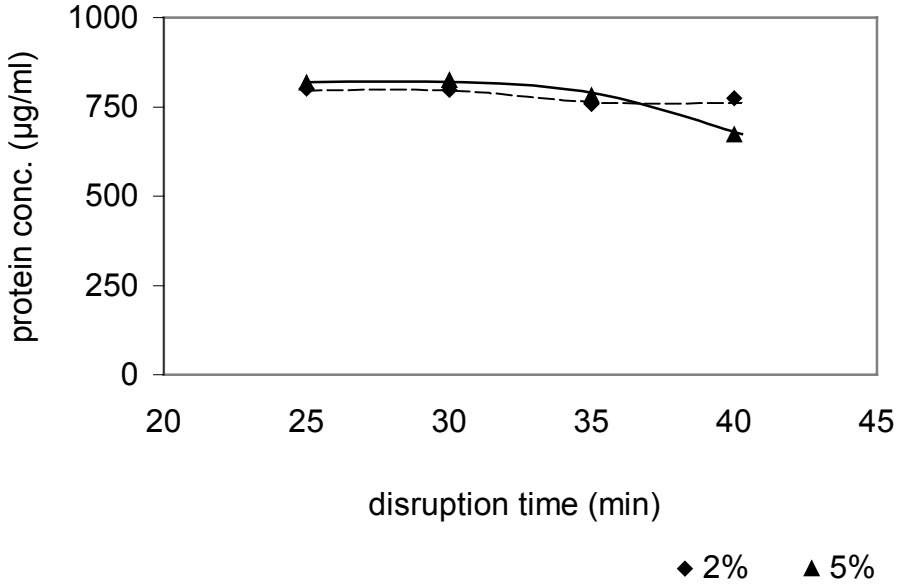


Figure 3.3. Time course of protein release for 2% and 5% cell suspension

Figure 3.3 shows that a maximum of 30 minutes is sufficient for a complete disruption and protein release. Once again, in the range of 2% to 5% cell suspension there is no difference, so is not necessary to use a higher concentration than 2% cell suspension.

To summarize, the following parameters were considered appropriate for cell disruption with a mixer-mill:

- 2% cell suspension
- 2:1 ratio of beads and cell suspension
- 40% vial loading (4 g cell suspension and 8 g beads (g/g))
- disruption time: 30 minutes

The standard disruption assay is described in 9.6.2.

Considering that in 30 minutes only two samples could be disrupted and larger volumes of culture broth are necessary to obtain the 2% cell suspension, for

routine analyses of enzyme activity in fermentation experiments, for instance, disruption with ultrasonics was performed.

3.3. Ultrasonics

The energy developed by ultrasound depends on resonance frequency of the device (15-25 kHz). Due to acoustic waves created by the probe vibration, air cavities are formed in the cell suspension. When these cavities are breaking down, the mechanical energy dissipated in the cell suspension is bigger than the cell elasticity and so the cell wall is destroyed *(Doulah, 1977)*. As for all mechanical procedures, the disruption kinetics is a first-order one. An important problem that has to be overcome is the temperature increase in the cell suspension during disruption.

3.3.1. Time Course of Enzyme Release

0.5 ml from a 2% cell suspension were centrifuged and the pellets were resuspended in 5 ml precooled NaCl solution (0.9%). During cell disruption, which lasted for 10 minutes, the sample was placed in an ice bath in order to prevent the inactivation of the enzyme due to temperature increase. Every minute, 500 µl from the sample were centrifuged and 100 µl from the supernatant were assayed for enzyme activity in order to investigate the time course of enzyme release from the cells. The DFAIII concentration obtained after performing the activity test can be directly correlated with the enzyme concentration and with the efficiency of cell disruption. The experiment was performed with *E.coli/*pMS*ift*OptWT. The activity test follows the standard procedure described in chapter 9.10.1.1. The results are shown in figure 3.4.

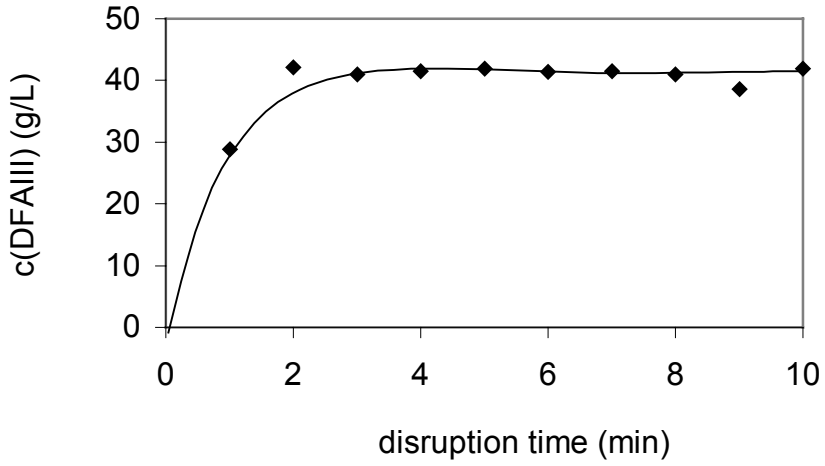


Figure 3.4. DFAIII concentrations as a function of time.

Figure 3.4 shows that 2 minutes of cell disruption are sufficient for an optimal enzyme release, because no higher DFAIII concentrations could be obtained after longer disruption time.

3.3.2. Validation of Cell Disruption Method

Due to its advantages (short time, small volume of cell suspension and simple handling procedure), ultrasonication cell disruption method was frequently used for standard analysis. The same tests were performed in order to verify if the disruption method gives reproducible results. Starting from a single 20% cell suspension, 9 identical samples were prepared and assayed for disruption as described in chapter 5.2. The experiment was performed with E. *coli/*pMS*ift*OptWT (DSMZ 13463) cells and the disruption of every sample lasted for 2 minutes. To investigate the released enzyme concentration an activity test was performed for each sample and the DFAIII concentrations obtained are summarized in table 3.2.

Table 3.2. Standard deviation for cell disruption by ultrasonics

Sample number	c(DFAIII) (g/L)	Standard deviation (%)
1	4.36	
2	4.31	
3	4.22	
4	4.18	
5	4.01	± 3.9
6	4.67	
7	4.22	
8	4.28	
9	4.45	

There were no significant differences between the DFA III concentrations obtained so the enzyme concentration was similar for all 9 samples. The standard deviation of 3.9% was considered acceptable, meaning that the cell disruption method offers reliable and reproducible results.

3.2.3. Comparison of Ultrasonics and Mixer-mill

Cell suspensions of different concentrations were assayed for disruption using both grinding with glass beads and ultrasonication methods. Because each method required different dilutions of cell suspension and the samples were prepared and handled differently, the DFA III concentration only is not sufficient in order to compare the two methods and so the enzyme activity had to be calculated for 1 liter culture volume. The results are summarized in table 3.3.

Table 3.3. Comparison ultrasonication and mixer-mill

Enzyme activity (U/L)		Coefficient
Mixer-mill	Ultrasonication	Ultrasonication/Mixer-mill
180,000	238,000	1.32
180,000	237,000	1.31
150,000	230,000	1.53
40,000	55,000	1.37

The protein release is comparable for both cases. Using the ultrasonication disruption, enzyme activities obtained are 1.4 times higher than those obtained by grinding with glass beads, which means that the disruption by ultrasonication is a slightly more efficient method than the grinding with glass beads.

The cell disruption using the ultrasonication had been shown to be a reliable and easy to perform laboratory procedure, it however cannot be scaled up when larger amounts of enzyme were required. In that case it was necessary to use another technical disruption method, the high–pressure homogenizer, for instance.

3.4. High-pressure Homogenizer

High-pressure homogenization is a method widely used for large-scale disruption. For disruption, a cell suspension is delivered by a piston pump into a homogenizing valve at a pressure higher than 50 MPa (500 bars). The cell disruption in a high pressure homogeniser also follows first-order kinetics and is

influenced by operating parameters such as homogeniser pressure, valve design, number of passes through the valve and cell concentration *(Schwedes and Bunge, 1992)*.

In order to obtain stock enzyme solutions, fermentation experiments were performed for *E.coli*/pMS*ift*OptR and pMS*ift*OptWT strains in a 20 L bioreactor. The pellets obtained after centrifuging the culture broth were resuspended in 0.04 M phosphate buffer pH 7 (see 8.6.3.) and investigated for cell disruption by a high-pressure homogenizer. This cell disruption was kindly carried out by the GBF laboratory. The operating parameters, established there were:

- 10% cell suspension concentration
- homogenizing pressure: 650 bars
- disruption time: 10 minutes
- 2 times passes through the valve assembly

The temperature recorded at the end of disruption was 30°C, so no deactivation of Inulase II could occur due to heating.
The enzyme activity obtained was 1.500.000 U/L for *E.coli*/pMS*ift*OptR and 230.000 U/L for *E.coli*/pMS*ift*OptWT, respectively, which is comparable to the one obtained for ultrasonication disruption, if all dilution factors are taken into consideration.

3.5. Discussion

Grinding with glass beads is a mechanical disruption method that was used for routine enzyme investigations. The efficiency of protein release depends on the disruption time, ratio of beads and cell suspension, cell concentration and the loading volume of the disruption vial. The influence of these parameters has been examined and it was found that an optimal disruption could be obtained after 30 minutes using a 2% cell suspension. These disruption parameters limited the applicability of the method for some experiments, such as fermentation in a 1 l bioreactor, because a large number of samples had to be handled in a short time and the sample volume had to be as small as possible. The necessity of using Benzonase to reduce the viscosity caused by nucleic acids is another disadvantage considering the enzyme price. The temperature increase is not significant, the highest temperature recorded at the end of disruption was 29°C and seemed to be primarily due to the environmental temperature, further increase was found to be in the range of 1°C per 5 minutes *(Hummel and Kula, 1989)*.

The ultrasonication cell disruption was adopted as a disruption method for routine analysis. As shown in figure 3.3, a complete release of protein could be obtained after 2 minutes and small only sample volumes are required. The acoustic waves output often reaches values of a few hundred watts. Because of the small volume of the fluid the temperature rises quickly making an intense cooling of the sample necessary. The problem was easily solved placing the sample into an ice bath during disruption. The disruption procedure is easy to perform and gives reliable and reproducible results (see table 2). The yield of released protein was found to be similar comparing the grinding and ultrasonication methods.

To obtain a bigger amount of stock enzyme solution, a high-pressure homogeniser was used. Large quantities of free protein could be obtained in short time (10 minutes) and because no adjacent dilution steps were performed,

the enzyme solution is more concentrated than the one obtained with the other two methods of cell disruption. The disruption efficiency is the same when enzyme activity of the culture is compared between ultrasonication and high-pressure homogenizer.

Chapter 4

Fermentation

4.1. Introduction

The fermentation research goal is to obtain the maximal amount of product in a given volume in the least possible time. To increase both cell density and productivity it is necessary to optimize the growth environment including the growth medium composition and physical parameters such as pH, temperature and agitation. A different approach may be to increase the amount of critical nutrients for growth, to prevent product degradation or to decrease the accumulation of toxic products *(Kleman and Strohl, 1994)*.

Escherichia coli is the most commonly used host for recombinant protein production mainly because it is a well-characterized system easily accessible to genetic modification and therefore suitable for recombinant protein production. From a nutritional point of view, it is a prototrophic bacteria due to its ability to thrive even on a simple medium containing a single compound as carbon and energy source and a few inorganic salts to supply the other essential elements required for growth. On the other hand, when performing high-cell-density fermentations of *E. coli* strains the inhibitory effect of some nutrients has to be taken into consideration, for example glucose (> 50 g/L), ammonia (>3 g/L), iron (> 1.15 g/L), magnesium (> 8.7 g/L), phosphorous (> 10 g/L) and zinc (> 0.038

g/L) *(Riesenberg, 1991, 1).* The most important by-product with inhibiting effect is acetic acid, which not only inhibits the cell growth; a correlation may exist between reduced recombinant protein production and acetic acid formation *(Bech Jensen and Carlsen, 1990, Brown et al., 1995).*

High cell density cultivation (HCDC) techniques for *E. coli* fermentation have been developed to improve productivity and to provide other advantages such as reduced culture volume, facilitated downstream processing, reduced wastewater, lower production costs and reduced investment in equipment (*Lee, 1996; Riesenberg et al., 1991, 2; Stratton et al., 1998)*. Since most proteins are intracellularly accumulated in recombinant *E.coli* strains, the productivity is proportional to the final cell density. Various high cell density fermentation techniques have been developed for growing *E. coli* strains in fed-batch cultures at cell concentrations higher than 100 g(DCW)L^{-1} *(Yee, 1992)*. The highest densities reported to date are 190 g(DCW)L^{1} for *E.coli* K12 *(Märkl et al., 1997)* and 175.4 g(DCW)L^{-1} for a recombinant *E.coli* XL-1 blue strain producing poly(3-hydroxybutyrate) *(Lee, 1994)* approaching the maximum cell concentration value of ~ 200 g(DCW)L^{-1}. This maximum value was stated considering that the viscosity of culture broth increases sharply when cell concentration exceeds 200 g(DCW)L^{-1} and almost loses the fluidity above 220 g(DCW)L^{-1} *(Mori et al., 1979)*.

For *E. coli/*pMS*ift*OptR strain producing Inulase II, a cell density of 10.5 g(DCW)L^{-1} was obtained *(Jahnz, 2001)*. The main goal of this project was to acquire higher activity for recombinant Inulase II implying high cell density cultivation of *E. coli/*pMS*ift*OptR strain.

Different cultivation strategies were adopted, investigating the cell development in different complex media at fermentation scales up to 20 L and appropriate feeding profiles. *E. coli/*pMS*ift*OptWT strain was also assayed for fermentation experiments in order to obtain a stock enzyme solution for further immobilization and enzyme characterization experiments.

The media and feeding solution (see 8.2.2) were appropriately chosen to meet the strain requirements. The cultivation processes at different scales are described in section 8.3.3.
Besides the *ift* gene coding for Inulase II, the cloning vector also contains a gene that confers ampicillin resistance to the host providing the possibility of plasmid selection. Therefore, the long and short time storage and all cultivations were performed in the presence of 60 µg/ml ampicillin solution.

4.2. LB Medium Fermentation

A first approach in obtaining Inulase II was to cultivate both *E. coli/*pMS*ift*OptR and *E. coli/*pMS*ift*OptWT strains in Luria Bertani medium containing 5 g/L yeast-extract, 10 g/L peptone from casein and 10g/L NaCl, pH 7 (see 8.2.2.1). This complex medium properly sustains the bacterial growth, the peptone and yeast-extract consisting of high amounts of amino acids, peptides, water-soluble vitamins and carbohydrates which ensure both the strain storage on petri plates and the culture promotion from single colony. Considering this, not only small-scale cultivation experiments were performed (5 ml, 500 ml and 1 L cultures) but also the LB medium was used to obtain the inocula for fermentations in other media.

4.2.1. Small Volume Cultures

To examine the bacterial development and the growth curve for small volumes LB medium fermentation in glass tubes cultures (5 ml) was carried out for:

- checking the strain viability for long and short time storage
- obtaining the inoculum for scaling up fermentation experiments since the use of late exponential growth stage cells is advisable.

Using single *E. coli/*pMS*ift*OptR colonies from LB medium-agar plates, reaction tubes containing 5 ml LB medium were inoculated and incubated at 37°C and shaken at 150 rpm. After 10 hours of incubation, culture broth aliquots were collected under sterile conditions and the optical density (OD) was measured at 600 nm against water. Plotting the OD against time, a growth curve was obtained (fig. 4.1).

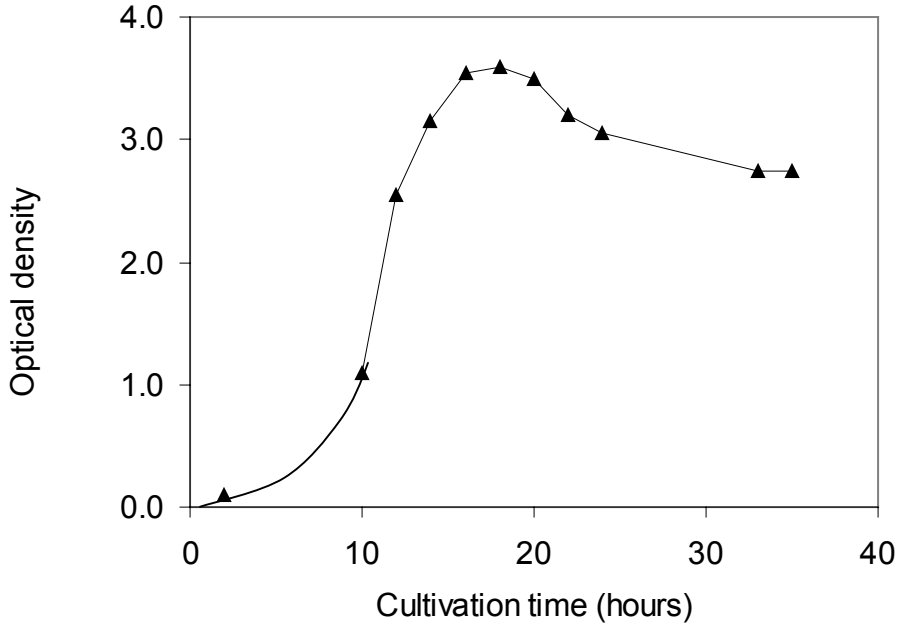


Figure 4.1. Growth curve of *E. coli/*pMS*ift*OptR strain in 5 ml LB medium (T=37°C, 150 rpm)

After 12 hours the end of logarithmic growth is reached and the stationary phase begins. After 22 hours a slow decline in OD is noticed. Repeated experiments lead to the same profile, and performing the activity test, an enzyme activity of about 350,000 U/L was obtained. 16 hours old cultures were considered appropriate to be used as precultures for scaling up experiments. To obtain shorter lag phases for some fermentation experiments, also 12 hours old precultures were used.

4.2.2. Cultivation in 500 ml Shake Flasks

A 500 ml main LB medium culture in a 2,5 L shake flask was inoculated in a ratio of 1:100 with preculture and incubated at 37°C and 150 rpm. After 16 hours of cultivation, the optical density, wet cell weight (WCW) and enzyme activity were determined. The results obtained for several experiments performed for both *E. coli/*pMS*ift*OptWT and *E. coli/*pMS*ift*OptR strains are summarized in table 4.1.

Table 4.1. Optical density, wet cell weight (WCW) and enzyme activity recorded for 500 ml LB medium cultures (*E. coli/*pMS*ift*OptWT and *E. coli/*pMS*ift*OptRM strains, T=37°C, 150 rpm, incubation time=16 h)

Culture	**OD** (600 nm)		**WCW** (g/L)		**Enzyme activity** (U/L)	
no.	**WT***	**RM****	**WT**	**RM**	**WT**	**RM**
1.	4.6	4.7	4.42	4.40	310,000	380,000
2.	4.7	4.6	4.62	4.46	350,000	385,000
3.	4.2	4.5	3.85	4.10	356,000	420,000
4.	4.8	5.0	4.43	4.54	300,000	360,000
5.	4.6	5.1	6.64	4.82	340,000	320,000

* *E. coli/*pMS*ift*OptWT

** *E. coli/*pMS*ift*OptR (R Mutant)

The optical density and consequently the wet cell weight recorded for *E. coli/*pMS*ift*OptR are only slightly higher than the similar values obtained for *E. coli/*pMS*ift*OptWT. A higher activity was expected (435,000 U/L) *(Walter, 2001)* for *E. coli/*pMS*ift*OptR enzyme compared with the activity of *E. coli/*pMS*ift*OptWT enzyme but only a 12% difference could be detected (~370,000 U/L for *E. coli/*pMS*ift*OptR and ~330,000 U/L for *E. coli/*pMS*ift*OptWT). No clear correlation

between the enzyme activity and the optical density could be stated, those cultures showing the highest OD do not have the highest enzyme activity. The enzyme activities values obtained after cultivating both recombinant *E. coli* strains in LB medium are lower than the ones obtained by Schubert for similar cultivation conditions *(Schubert, personal communication)*.

- **Induction with IPTG and Lactose**

The ift gene coding for inulase II is cloned in a pUC 19 vector, the expression is controlled by a *lac*-based promoter. The gene expression gene can be induced either with IPTG (isopropyl-β-D thiogalactopyranoside) (the synthetic inducer of the Lac operon) or with allolactose (the natural inducer of the operon) *(Matthews and Nichols, 1998)*. IPTG is used at laboratories scale because in contrast to lactose, the IPTG cannot be hydrolyzed and broken down by the cell. Hence, its concentration does not change during an experiment and leads to an efficient induction. For large-scale production the use of IPTG is undesirable because of its price and toxicity which complicates the downstream and wastewater processing *(Makrides, 1996)*.

Trying to increase the Inulase II concentration, both IPTG and lactose inductions were performed in 500 ml LB medium shake flasks experiments (for solutions concentrations and the cultivation procedure – see 9.3.2.3). To insure a maximal enzyme production, IPTG and lactose were added in the late log phase of culture growth curve (after 12 hours of incubation), not to disturb the cultures development but only to induce a better expression of the plasmid.

The changes in culture development and enzyme activity following the induction were investigated collecting culture broth samples before and after induction (after 12 hours and after 16 hours of incubation, respectively). The small differences recorded for optical density between 12 and 16 hours certified a normal culture promotion after induction. The enzyme activity tests were also carried out and the values obtained after induction were only slightly enhanced

compared with the one recorded before induction, but not significantly to be due to an induction process. The recombinant inulase II is constitutively expressed without the necessity of induction (*Schubert, personal communication)*. This statement could be certified by the fact that a significant increase in enzyme activity could not be detected after the addition of IPTG or lactose.

Further experiments were carried out in fermenters, in order to increase the enzyme production by more suitable and controlled environment.

4.2.3. Cultivation in 1 L Bioreactor

The cultivation of *E.coli/*pMS*ift*OptR bacteria cells in LB medium was performed in 1 L bioreactors, with a maximum working volume of 700 ml. Compared with shake flasks experiments, it was expected to enhance the cell density and consequently the enzyme activity due to the improved aeration and pH control. The bioreactor preparation and the experimental conditions are described in 8.3.3.2.1. 7 ml from the same second preculture (100 ml LB medium, incubated for 12 hours in standard conditions) were used to inoculate each bioreactor (the experiment was performed in parallel in four identical bioreactors). The oxygen saturation dropped to 40% after about 5 hours of fermentation and then was maintained constant due to the cascade control system that correlates the dissolved oxygen concentration in the culture broth with the stirring system and reactor aeration. The pH was kept at constant value (pH 7) without necessity to recourse to significant volumes of correction solutions. The culture development was monitored measuring the optical density. The recombinant inulase II activity was followed and the results are presented in figure 4.2. To avoid drastical modifications of the culture volume, the wet cell weight (WCW) and dry cell wet (DCW) were not investigated. The small culture broth volumes that could have been collected, without significantly perturbing the cultivation, would have not ensured a reliable value for WCW and DCW.

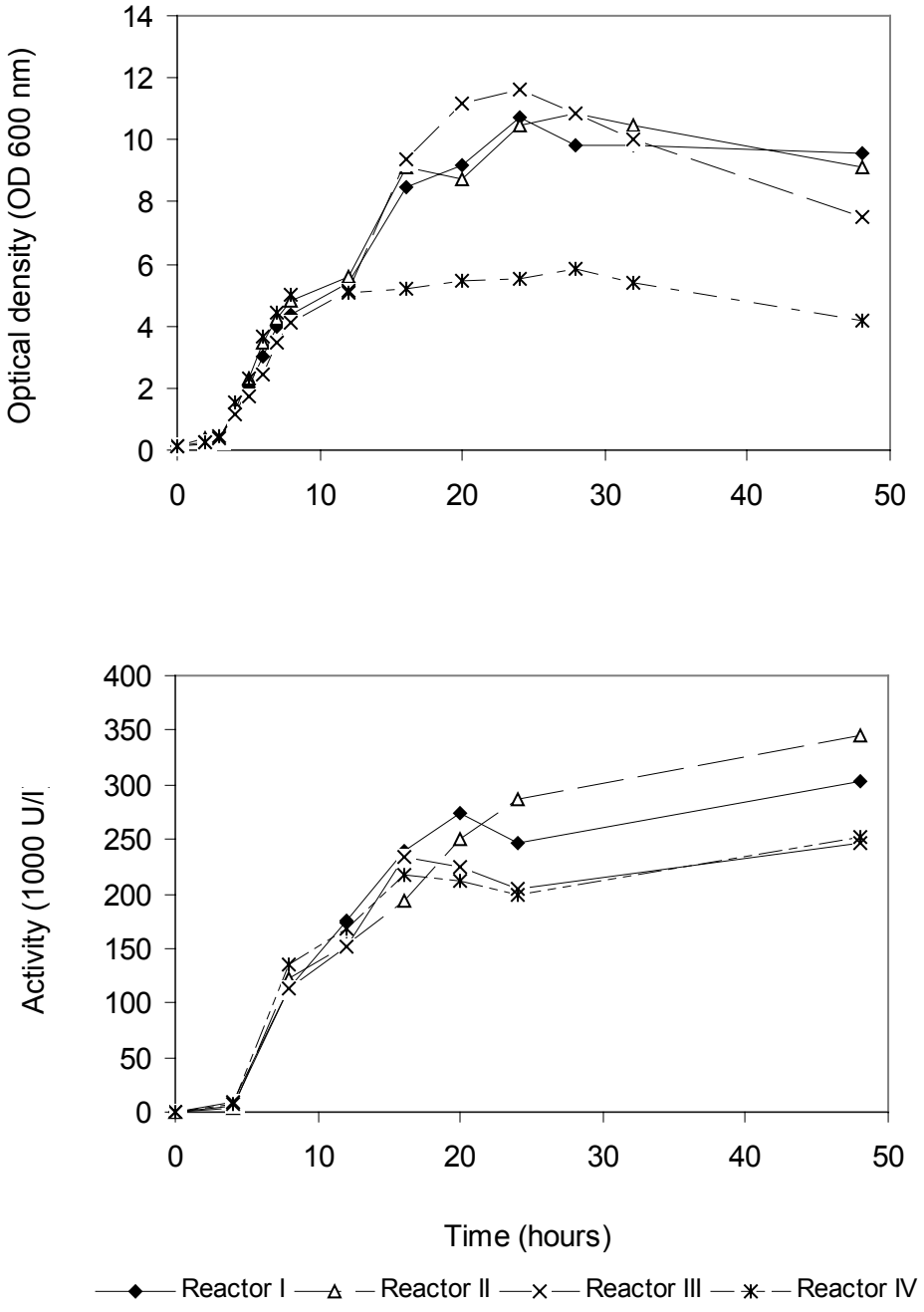


Figure 4.2. The time courses of optical density and enzyme activity for 1 L bioreactor with LB medium fermentation experiments. (*E. coli/*pMS*ift*OptR strain, T=37°C, 40% oxygen saturation)

After a short lag period (of 4 hours) all four cultures developed similarly for another 8 hours. An important difference in the growth curve profiles was recorded after 12 hours of fermentation, when culture IV entered in the stationary phase, while the other three cultures continued the exponential growth, but at a reduced growth rate. This difference was not perceived at enzyme activity level, the values recorded being comparable for all cultures. Except culture IV, there is a good correlation between the optical density and the enzyme activity until 25 hours of fermentation (OD ~ 12 and ~ 250,000 U/L enzyme activity). In the final period of the experiment, the enzyme activity slightly increased although the optical density of the cultures decreased. Highest activity values around 300,000 U/L were achieved after 48 hours.

Enzyme activities tests were also performed to check the presence of Inulase II in the culture broth, as a consequence of cell lyses. A linear increase for all four cultures could be detected while the experiment proceeded; the highest values were recorded at the end of fermentation but did not exceed 10% of intracellular activity.

Since no improved results were obtained for this complex medium as compared with shake flasks cultivations, different approaches had to be adopted, investigating other media in fermentation experiments involving appropriate feeding strategies.

4.3. Yeast-extract / Glycerol Medium Fermentation

Yeast-extract- glycerol medium was considered to sustain the recombinant *E.coli* growth in fed-batch fermentation experiments *(Jahnz, 2001)*. Using LB medium second precultures, fermentation experiments were performed in 1 L and 20 L bioreactors. The feeding solutions contained glycerol and yeast-extract and the fed-batch strategy followed the glycerol depletion, taking into consideration that growth-inhibiting acidic by-products, as acetic acid, are produced in response to

oxygen limitation or excess carbon *(Korz et al., 1995)*. The medium and the fed batch solutions are presented in 8.2.2.2, the bioreactors preparation and the experiment conditions - in 8.3.3.2.1. (1 L bioreactor) and 8.3.3.2.2. (20 L bioreactor).

4.3.1. Cultivation in 1 L Bioreactor

The starting volume for this experiment was 600 ml, because 2 feeding steps (each time 50 ml) were taken into consideration. The cultivation was performed in 3 parallel bioreactors, inoculated with 6 ml culture broth out of a 16 hours old 100 ml LB medium second preculture. The fermentation conditions were identical for all 3 bioreactors. The cell development was followed measuring the optical density (OD), and the glycerol consumption. Activity tests were performed in order to determine the recombinant inulase II activity. In figure 4.3, the optical density variation and the enzyme activity are plotted against time for all 3 reactors.

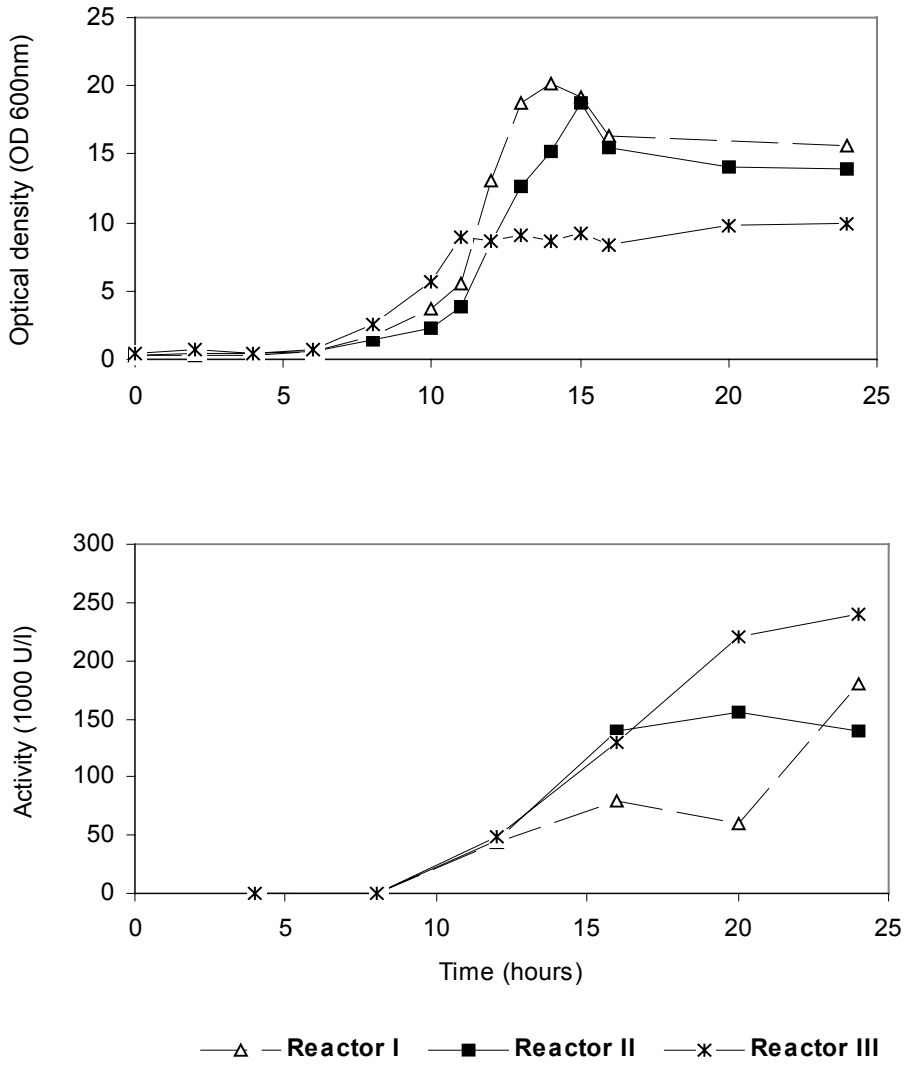


Figure 4.3. The time courses of optical density and enzyme activity for 1 L bioreactor yeast-extract- glycerol medium fermentation experiments. (*E. coli/*pMS*ift*OptR strain, T=37°C, 40% oxygen saturation)

As in can be seen in figure 4.3, after a relatively long lag period (~ 8 hours) the cultures started to develop differently. 2 out of 3 cultures had an exponential growth phase, which lasted until 12 h of fermentation, while the growth stopped for culture III after 12 hours. Towards the end of experiment, the optical density recorded for reactor III enhanced, but not significantly. From enzyme production

point of view, it is interesting to notice that the highest activity (~250,000 U/L) was detected for the culture with the lowest OD (reactor III).

The glycerol and acetate concentrations were monitored (figure 4.4) during the fermentation, these parameters being particularly important for the proceeding of the cultivation: the glycerol concentration determined the fed-batch profile and the acetate concentration could perturb the cultivation (details in Discussion section, page 73).

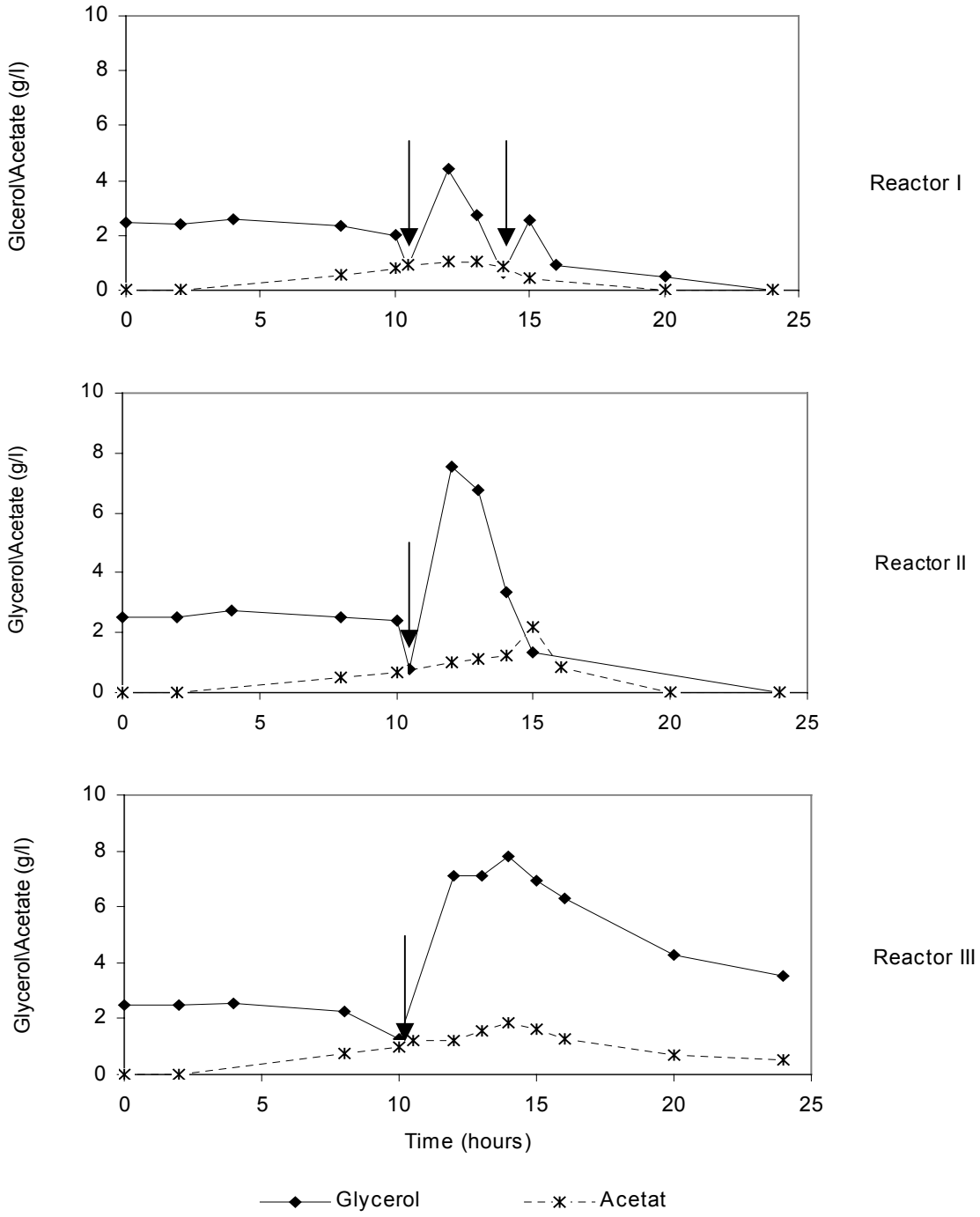


Figure 4.4. The time courses of glycerol and acetate concentrations for 1 L bioreactor yeast-extract- glycerol medium fermentation experiments. (*E. coli*/pMS*ift*OptR strain, T=37°C, 40% oxygen saturation). At arrows, feeding solutions were added.

The feeding was performed according to the glycerol depletion from the culture medium. After 10 hours of fermentation, the glycerol concentration reached values close to zero, and so a first feeding step was performed. The feeding solution (50 ml) contained 100 g/L yeast-extract and 50 g/L glycerol. A second feeding step (50 g/L glycerol in 50 ml) was performed for reactor I after 14 hours of fermentation, when glycerol concentration reached again a minimal value. Until the end of the experiment, cultures I and II succeeded in the complete metabolization of the administrated glycerol, while a high concentration (~ 4 g/l) was still recorded after 24 hours of fermentation for culture III. The optical density recorded for this culture also reflected the slow glycerol consumption.

Acetate concentration did not reach values high enough as to inhibit the culture development or the enzyme production (inhibitory acetate concentrations are considered to be ~10 g/L *(Pan et al., 1987, Riesenberg, 1991.1))*. The highest values were recorded when the glycerol concentration was also high, immediately after the feeding.

High glycerol concentration, although sustaining the cell development, seemed not to enhance the enzyme production. At this point, a continuous feeding profile was considered to be more suitable to reach higher enzyme activity and also to prevent the acetate accumulation in the culture medium. The bioreactor volume (1 L) prevented a continuous fed-batch experiment, so for further experiments a 20 L scale-up cultivation was adopted.

4.3.2. Cultivation in 20 L Bioreactor

A fed-batch experiment with yeast-extract- glycerol medium was performed in a 20 L bioreactor, having a starting volume of 10 L. To reduce the lag phase, the inoculation was made with a 12 hours old second preculture (100 ml, LB medium). Except for the feeding procedure, the fermentation conditions were similar to the cultivation in 1 L bioreactor. Foam was suppressed by the automatically addition of sterile Ucolub N 115 as antifoam agent. The time course

of fermentation parameters is shown in figure 4.5. After a 6 hours lag period the culture entered the exponential growth phase and the glycerol consumption increased. In the same time, the oxygen demand increased and the 40% saturation could only be maintained operating at high agitation speed (600 rpm). After 12 hours of fermentation, the glycerol was depleted and the feeding procedure started. The 500 ml fed-batch solution containing 100 g yeast-extract and 50 g glycerol was intended to be continuously pumped into the bioreactor, to avoid an excess of glycerol and consequently the acetate accumulation in the culture medium. The feeding proceeded for only one hour, because the glycerol concentration increased rapidly and remained around 3 g/l meaning that the feeding profile exceeded the uptake capacity of the culture. In addition, the oxygen saturation dropped to zero and even the high rotation and the maximum opening of the aeration valve could not insure the culture oxygen demands. Acetate formation was not detected and other 2 punctual administrations of feeding solution (100 ml solution containing 50 g glycerol) were carried out only when the glycerol was completely depleted from the culture medium (after 15 and 20 hours of fermentation, respectively).

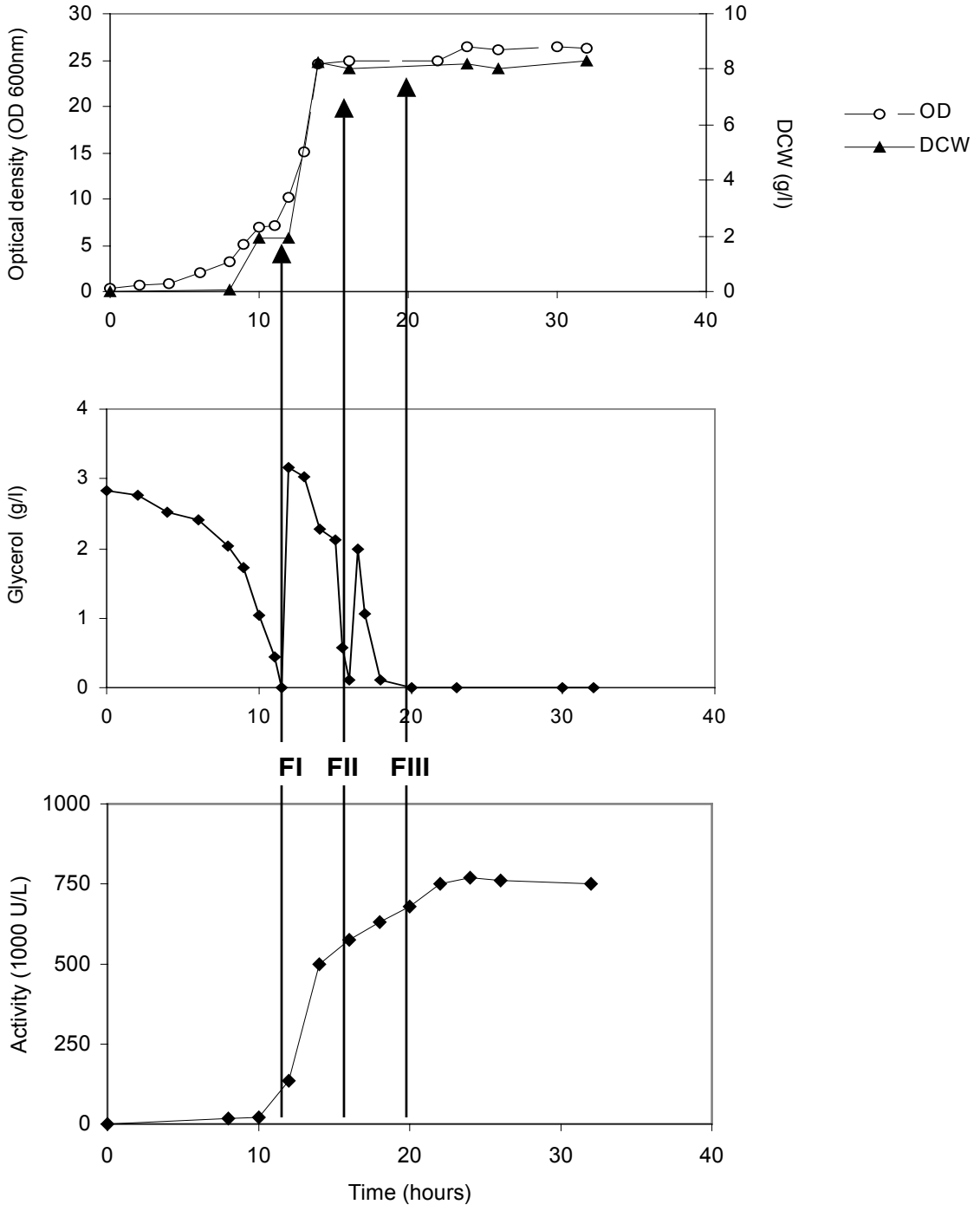


Figure 4.5. Time-courses of optical density, DCW, glycerol concentration and enzyme activity for the 20 L bioreactor yeast-extract-glycerol medium fermentation experiment (*E.coli/*pMS*ift*OptR strain T=37°C, 40% oxygen saturation). At arrows, fed-batch solutions were added.

The enzyme activity was monitored every 2 hours, and a good correlation was noticed between the dry cell weight increase and the enzyme production (see fig 4.5). The maximum value for enzyme activity (750,000 U/L) was determined after 22 hours of fermentation and remained constant until the end of the experiment, following the same profile as the OD and DCW variations.

The presence of the recombinant inulase II as free enzyme in the culture medium due to cell lysis was investigated assaying the centrifuged culture broth to activity tests. A constant value of 20,000 U/l was recorded after 18 hours of fermentation, representing only around 3 % as compared to the intracellular enzyme activity.

Other fermentation experiments were performed following the same strategy, adjusting only the feeding rate at different values. Employing the yeast-extract-glycerol medium at 20 L bioreactor cultivation scale, no higher values than 750,000 U/L and ~ 10 g/L were obtained for the enzyme activity and DCW, respectively.

Therefore other media were tested for their suitability to increase volume activity and DCW.

4.4. Mineral / Yeast-extract Medium Fermentation

Fermentation experiments were also performed in a 20 L bioreactor using a mineral-yeast-extract medium. The medium composition and the fermenter preparation are described in chapter 8.2.2.4 and 8.3.3.2.2, respectively. Because of the high amount of yeast-extract in the starting medium, 5 ml antifoaming agent were added before fermenter sterilization. As inoculum a 12 hours old modified LB medium culture (see 8.2.2.2 for medium description), was used. The absence of the glycerol from the starting medium required another control parameter for the feeding profile, which was the concentration of dissolved oxygen. (8.3.3). The continuous fed-batch strategy was intended to ensure a 30% oxygen saturation and was set to start later than 10 hours, when the

consumption of the initial carbon source (yeast-extract) was indicated by an increase of the dissolved oxygen saturation.

The fed-batch procedure started after 14 hours of fermentation; the solution containing 600 g/L glycerol was slowly pumped into the fermenter to obtain a carbon-limited cultivation and consequently to induce a reduced growth rate. Since the glycerol concentration remained below the detection limit during the whole fermentation process it was assumed that the carbon source met the culture requirements and was efficiently metabolized. Therefore, the acetate formation was efficiently circumvented; no acetate could be detected in the culture supernatant. The optical density, enzyme activity and dry cell weight were also monitored during the whole experiment and are represented in figure 4.6 as a function of time.

Between 10 and 14 hours, the culture developed exponentially at a high growth rate (μ=0.20) and the changes recorded in μ after 14 hours are the result of the feeding profile (see figure 4.6). A significant increase was recorded in the dry cell weight level, accordingly to the optical density increase.

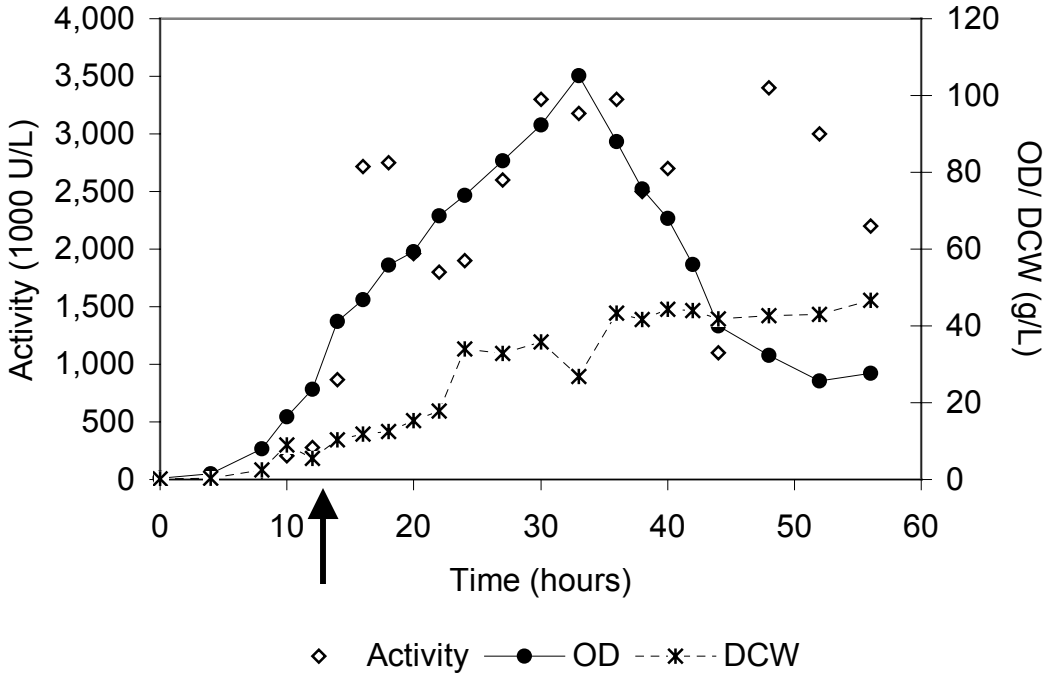


Figure 4.6. Time-courses of optical density, DCW and enzyme activity for 20 L bioreactor mineral -yeast-extract medium fermentation experiment (*E.coli/*pMS*ift*OptR strain T=37°C, 30% oxygen saturation). At arrow, fed-batch process was initiated.

An inexplicable variation was observed for enzyme activity which is not due to analytical problems (the values being determined repeatedly): high values are obtained after around 16, 30 and 48 hours of fermentation. Inbetween significant losses in the enzyme activity occurred, reaching occasionally even a 70% decrease. This lack of activity could not be explained by cell lysis, since the values recorded for free enzyme in the culture broth only represent 3% of the missing enzyme. To maintain the pH level, around 200 ml from a 10% NH_3 solution were automatically pumped into the fermenter. In the same time, an important volume of antifoaming agent was also required. The profile of the optical density variation could be drastically influenced by the amount of antifoam agent, the reliability of the high values recorded, up to 100 units, therefore being questionable. Till the end of the fermentation the optical density decreases very rapidly, although the dry cell weight shows a tendency to increase.

Because of the tight time schedule, only one more fermentation experiment was performed to investigate this medium. The culture development and the whole experiment course were completely different this time, including the results for enzyme activity, optical density and dry cell weight being significantly lower. It will be necessary to carry out some more experiments in order to obtain reliable information and to elucidate the question marks raised during recombinant *E. coli* fermentation in mineral-yeast extract medium.

Investigating the possibility that the cultivation medium is responsible for the lack of activity recorded to the end of fermentation, cultivation experiments were carried out in a different medium.

4.5. Mass-culture Medium Fermentation

Mass culture medium fermentation experiments were carried out in 1 L and 20 L bioreactors for both *E. coli* strains investigated in this work. The composition and preparation of the medium is based on published data *(Riesenberg et al., 1990)*, but some modifications were made (see 8.3.3). The medium was considered to insure a proper balance between the carbon and nitrogen sources and to meet the trace elements requirement of *E. coli* cells which can be critical for a high-cell density fermentation especially when recombinant proteins are expressed *(Horn et al., 1996)*. Exponentially grown cultures (mass-culture medium, 12 hours old second preculture) were used as inoculum. The fermentations were carried out at 37°C and the oxygen saturation was kept at 20% saturation by a pO_2-agitation rate-aeration valve control loop.

4.5.1. Cultivation in 1 L Bioreactor

Some 1 L bioreactor fermentations experiments of *E. coli/*pMS*ift*OptR strain in mass-culture medium were carried out under standard conditions (8.3.3.2.1). The feeding procedure was performed according to the glycerol depletion from the culture medium. 50 ml fed-batch solution containing 10 g/L glycerol and 2.5 g/L yeast-extract (ratio 4:1 between glycerol and yeast-extract) were manually injected in the reactors II and III each time when the glycerol concentrations recorded for this cultures were close to zero (after 14, 15 and 23 hours, respectively). No feeding profile was adopted for reactor I, in order to compare the culture development in batch and fed-batch conditions. During cultivation, the optical density, glycerol and acetate concentrations and enzyme activity were determined and represented as time functions in figure 4.7.

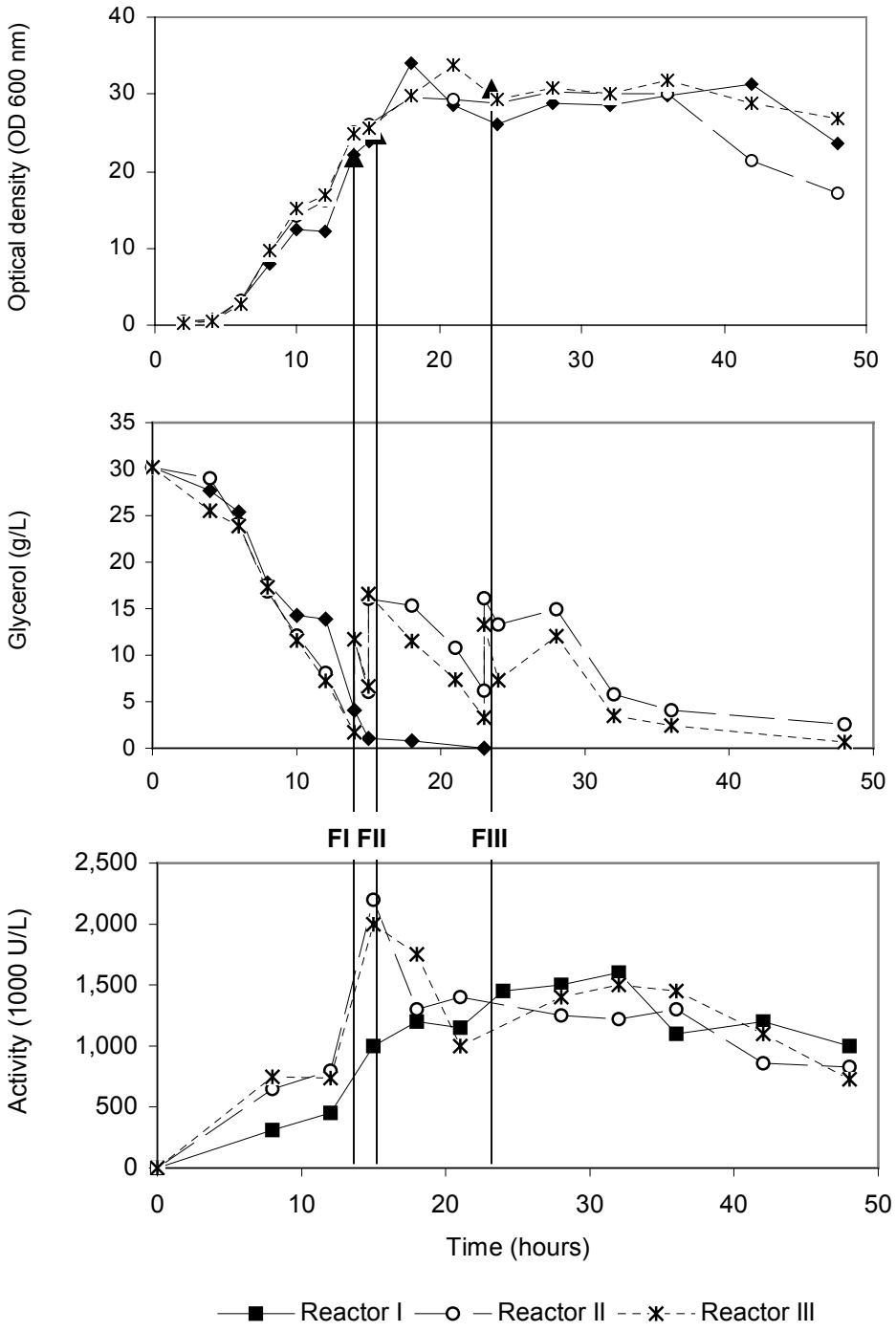


Figure 4.7. The time-courses of optical density, glycerol concentration and enzyme activity for a 1 L bioreactor mass-culture medium fermentation experiment (*E.coli*/pMS*ift*OptR strain T=37°C, 40% oxygen saturation). At arrows, fed-batch solutions were added.

The cellular grow is similar for batch and fed-batch cultures. An optical density around 30 was recorded for all three cultures after 20 hours of fermentation, when the stationary phase was reached. Although another fed-batch solution was administrated to cultures II and III, no further enhance of the optical density could be determined. The glycerol consumption occurred every time with the same rate, but no significant changes were detectable at optical density level. A possible inhibition due to the metabolic by-product acetate was excluded since it accumulated only up to 4.5 g/L, throughout the whole fermentation, which is bellow the inhibition limit (10 g/L) *(Riesenberg, 1991, 1)*.

The intracellular enzyme activities increase sharply for cultures II and III after the first feeding step, reaching ~2,000,000 U/L, 50% higher than the value recorded for culture I at the same fermentation time. Strangely, after a second feeding step the enzyme activities in both cultures dropped to 75% of the value recorded before feeding. A small increase was achieved for culture III after a third feeding step, while the enzyme activity in culture II remained almost constant until 38 hours of fermentation. From this time further, the activities recorded for all three cultures differ only little one from the other, the values covering a domain between 1,000,000 and 1,500,000 U/L. For the last 10 hours, the glycerol consumption rate decreased drastically, and the enzyme activities for all three cultures recorded a declining tendency. Final values of ~ 1,000,000 U/L were determined for the enzyme activity, independent on the cultivation strategy.

The loss of activity recorded after the second feeding step was investigated and some possible explanations will be given in the Discussion section, page 73.

The activity values reached with this medium are the highest obtained so far (2,000,000 U/L), but a 25% decline could be observed till the end of experiment. For future cultivation, a different feeding profile should be tested.

4.5.2. Cultivation in a 20 L Bioreactor

Fermentation experiments using the mass-culture medium were carried out at the 20 L bioreactor scale, which offers the possibility of performing different feeding profiles. The excess of carbon source can lead to the formation of growth-inhibiting metabolic products such as acetic acid and so it is important to maintain the control over the cellular growth. Different approaches were investigated:

- a continuous feeding rate was adopted to maintain the carbon-limited growth during the fed-batch and
- an exponential feeding rate was assayed for maintaining the growth rate at a pre-determined value, lower than the maximum growth rate possible for this *E. coli* strain.

4.5.2.1. Continuous Feeding

The 20 L bioreactor containing mass-culture medium (for medium composition and bioreactor preparation, see 8.3.3) was inoculated with 100 ml culture of late log phase (a 12 hours old mass-culture medium second preculture). The feeding solution contained 20 g/L glycerol and 5 g/L yeast-extract (ratio 1:4) and the feeding adopted for this experiment was meant to start after 16 hours of fermentation (considering the results from the previous experiment, performed in 1 L bioreactor, see figure 4.7) when the glycerol concentration of the culture broth was expected to be minimal. The dissolved oxygen control was realized using the pO_2-agitation speed-aeration valve control loop, which maintained a value around 20% for oxygen saturation in the culture broth during the fermentation experiment.

The optical density, inulase II activity, glycerol and acetate concentrations and dry cell weight were monitored during the whole experiment and are represented in figure 4.8 as time functions.

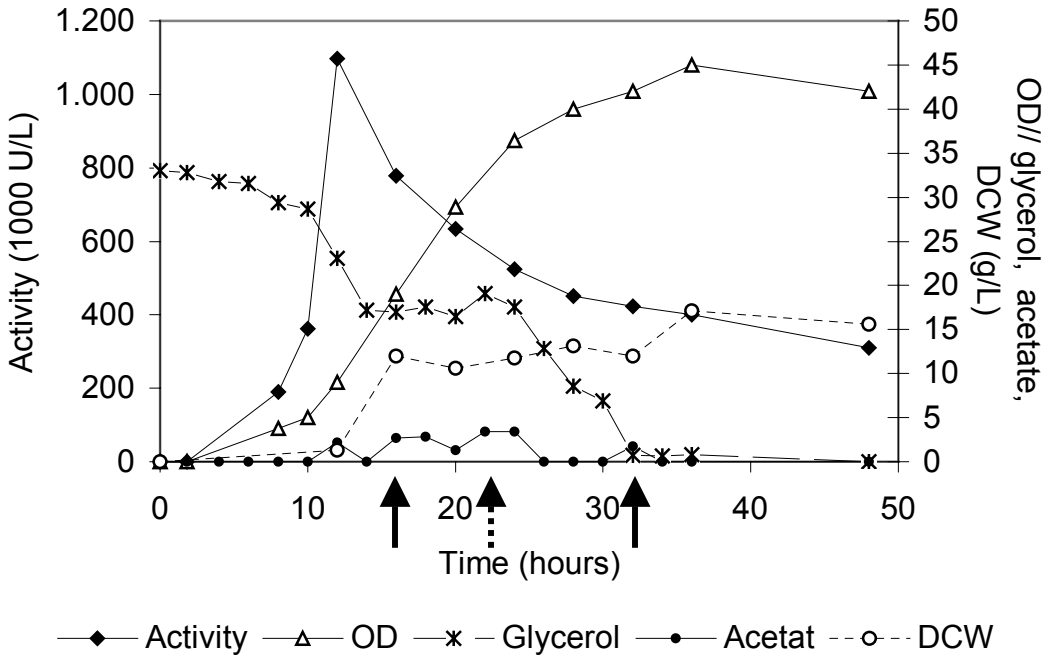


Figure 4.8. The time-courses of optical density, enzyme activity, glycerol, acetate concentrations and DCW for 20 L bioreactor mass-culture medium fermentation experiment (*E.coli/*pMS*ift*OptR strain, T=37°C, 20% oxygen saturation). At arrows, the fed-batch process started. At dotted arrow, the feeding was stopped.

Considering the results from the fermentaton in 1 L bioreactor, when the glycerol depletion from the culture medium occurred after 14 hours of fermentation, the fed-batch procedure was set to start after 16 hours. As it can be seen in figure 4.8, the exponential growth phase started for this culture after 10 hours, with a 2 hours delay towards the fermentation in 1 L bioreactor. After the initiation of the feeding procedure, the glycerol concentrations remained at high level (around 20 g/L), the feeding rate exceeded the culture metabolization capacity. The cell development continued at high growth rate since no carbon limitation was achieved. The glycerol excess though did not lead to the accumulation of high acetate amounts in the culture medium, the highest values recorded (around 4

g/L) being below the inhibition limit *(Riesenberg, 1991, 1).* In addition, no inhibition could be detected at the optical density level. The continuous fed-batch process was allowed to proceed for 6 hours and since no decrease of the glycerol could be detected the feeding was stopped after 22 hours of fermentation. After this, the growth continued but at a reduced rate and consequently the glycerol depletion was accomplished after 32 hours of fermentation, when a second fed-batch procedure was attempted. This time the carbon source supplied in the medium with the feeding solution was successfully metabolized and no accumulation of glycerol occurred but also no further enhance of the optical density could be obtained. The profile of the dry cell weight variation curve recorded during this experiment matches with the optical density variation until 16 hours of fermentation. In the following period, the exponential growth certified by the optical density variation continues, though constant values are recorded for the DCW. To the end of cultivation, 15 g/L dry cell weight were obtained, corresponding to an optical density around 40.

The enzyme activity was also followed during the fermentation process, the highest value (~ 1,100,000 U/L) being recorded after 12 hours of fermentation before the initiation of the fed-batch procedure. After this moment, even though the culture continued to develop, a sharp decrease of the enzyme activity was recorded when the glycerol concentrations in medium exceeded the strain necessities. The loss of activity continued, but at a reduced rate, even in the latest stage of experiment, when the glycerol was efficiently metabolized. To the end of fermentation, a 70% decrease was detected, meaning that only an activity of 310,000 U/L could be recovered from the fermentation broth. This loss of activity could have been a consequence of the cell lysis, therefore centrifuged medium aliquots were assayed for activity tests, expecting to find a significant part from the 70% intracellular enzyme lacking to the end of fermentation as free enzyme in the culture broth. No higher values than 70,000 U/L were obtained, representing no more than 7% towards the intracellular enzyme.

No improvement compared with the 1 L bioreactor experiment with identical medium was achieved. Even if a high an activity of 1,100,000 U/L were obtained

after 12 hours, only 310,000 U/L could be recovered at the end of experiment. The fed-batch process was not performed at desired parameters and so a dry cell weight of only 15 g/L was reached. Trying to prevent the problems encountered with this experiment, another feeding profile was approached for the 20 L fermentation in mass-culture medium.

4.5.2.2. Exponential Feeding

The exponential feeding is considered to be the most successful fed-batch strategy allowing the cells to develop at a constant growth rate below the critical rate at which acetate if formed, by limiting the carbon source concentration and the excess of dissolved oxygen in the culture broth *(Strandberg, 1991)*. This fermentation experiment was kindly carried out by GBF laboratory, since at that time the ZI equipment could not sustain an exponential feeding procedure.

An exponential feeding profile was investigated in the frame of recombinant *E. coli* cultivation, aiming to achieve a high-cell density fermentation. The yeast-extract and glycerol concentrations in the mass-culture medium recipe were modified, starting the cultivation with 2 g/L yeast-extract and 6 g/L glycerol (ratio 1:3, instead of 1:4 for previous experiments). This medium composition was considered to insure the strain necessities for nitrogen and carbon source in the batch phase *(Ross, personal communication).* 100 ml second preculture (mass-culture medium, incubated for 12 hours in standard conditions) were used to inoculate the reactor. During the exponential feeding phase, 2 L solution containing 400 g glycerol and 100 g yeast-extract were pumped into the fermenter. The feeding parameters were calculated using the equations 2.1 and 2.2 (see 2.1). A maximal possible growth rate of μ=0.25 was calculated for this *E. coli* strain. In order to obtain reduced growth rate (μ=0,12) compared with the one calculated from previous experiments (μ=0,25), the feeding rate was set to increase from 16.5 ml/h/$L_{culture}$, at the beginning of feeding process, to 256,5

ml/h/$L_{culture}$ through the end of the experiment maintaining a 20% oxygen saturation in the cultivation medium.

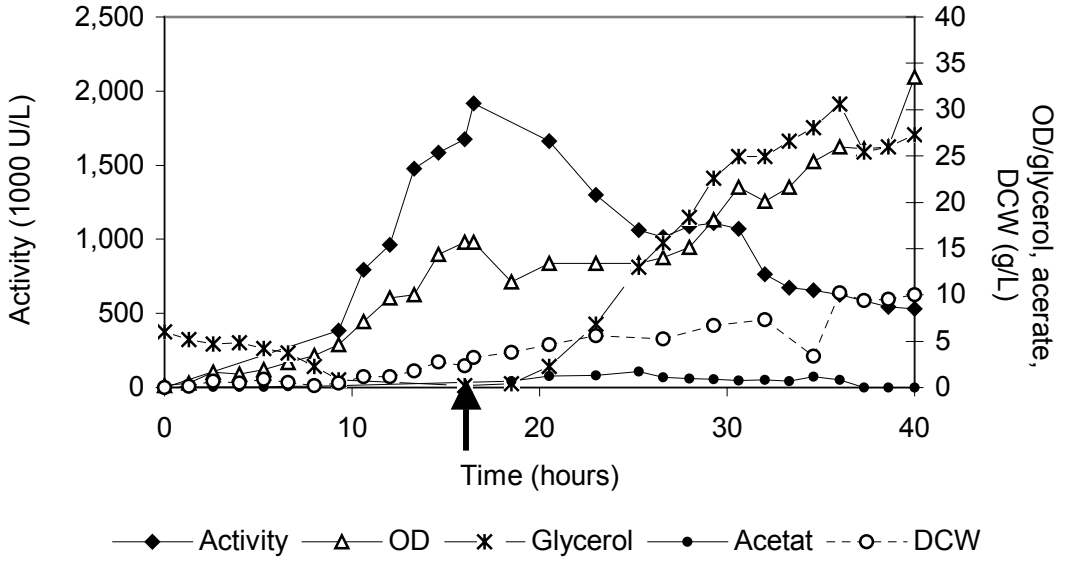


Figure 4.9. The time-courses of optical density, enzyme activity, glycerol, acetate concentrations and DCW for 20 L bioreactor mass-culture medium fermentation experiment (*E.coli/*pMS*ift*OptR strain, T=37°C, 20% oxygen saturation). At the arrow, the fed-batch process started.

The glycerol depletion occurred after 10 hours from the beginning of cultivation. After an unexpected long batch phase, which lasted 17.5 hours, the fed-batch procedure was initiated as a response to reaching the threshold value of 20% dissolved oxygen concentration recorded by the pO_2-agitation speed control unit. Only for 2 hours the culture was able to metabolize efficiently the nutrients that were supplied with the feeding solution, a fact confirmed by the absence of glycerol from the culture medium. After that time the glycerol-feeding rate exceeded the strain necessities and glycerol started to accumulate in the medium, though the reduced growth rate is achieved and acetate concentration in the culture medium is not significant, far from the inhibition limit. The optical

density increase was mirrored in the dry cell weight level (around 10 g/L were obtained through the end of experiment), but not at the enzyme activity level. After a high value recorded immediately after the fed-batch procedure started (~ 2,000,000 U/L), a rapid loss occurred. In less than 8 hours 50 % of inulase acivity was recorded. For the last 15 hours of cultivation the decrease continued but at lower rates, in the end of the fermentation only 550,000 U/L could be obtained. Investigating the presence of recombinant inulase II as free enzyme in the culture medium as a result of cell lyses, 80,000 U/L could be detected. This value covers only 5% from the lacking enzyme activity.

Adopting the exponential feeding profile, a lower growth rate could be induced comparing with the one from continuous feeding profile experiments even if the glycerol exceeded the culture necessities. Still, the loss of activity could not be circumvented and also no high cell densities were obtained. Some possible explanation regarding the lack of activity at the end of fermentation will be given in the Discussion section.

4.5.3. Cultivation in 20 L Bioreactor to Obtain Stock Enzyme Solutions

Some fermentation experiments were performed for both *E. coli/*pMS*ift*OptR and *E. coli/*pMS*ift*OptWT strains, aiming to obtain recombinant enzyme solutions with a high activity for immobilization. The fermentation experiments were carried out in mass-culture medium, being the most investigated medium and with the best results, at 20 L bioreactor scale.

*A. E. coli/*pMS*ift*OptR strain

The results obtained from previous *E. coli/*pMS*ift*OptR strain fermentation experiments in mass-culture medium showed that:

- a high enzyme activity is obtained after around 12 hours of fermentation and

- through the end of cultivation the activity decreases to less than 50%, without an obvious reason, independent of the fed-batch strategy. A possible reason for this may be the presence of proteases which degrade the recombinant inulase (see Discussion, page73).

These considerations led to adopt a different fermentation strategy: the cultivation was stopped after 12 hours to prevent the loss of activity and the *E. coli* proteases that could have possibly destroyed the enzyme were inactivated heating the culture broth at 60°C for 30 minutes. The recombinant inulase II is stable up to 65°C so the fermenter heating will not affect the enzyme.

The experiment was performed in standard conditions; the ratio between the glycerol and yeast-extract in the starting medium was the same as for the exponential feeding experiment (see 4.5.2.2). The cultivation course followed the profile described for exponential feeding fermentation experiment. After 12 hours of cultivation the bioreactor temperature was raised to 60°C for 30 minutes. Investigating the enzyme activity no enzyme denaturation could be identified as a consequence of temperature increase. The pellets were further isolated from the culture medium by centrifugation and to obtain the stock enzyme solution, the cell disruption was performed using a high-pressure homogenizer (see 8.6.3.). As a result of this experiment 1,5 l enzyme solution with an activity of 1,500,000 U/L were obtained.

B. E. *coli/*pMS*ift*OptWT Strain

The E*. coli/*pMS*ift*OptWT strain was assayed for cultivation in mass culture medium. The goals of this experiment were:

- to investigate the strain behavior in fermentation experiments and
- to obtain a stock enzyme solution necessary to immobilize and characterize the E*. coli/*pMS*ift*OptWT enzyme solution.

The fermentation procedure was similar as the one for the continuous fed-batch fermentation of E*. coli/*pMS*ift*OptR in mass-culture medium. The continuous

feeding profile was set to maintain carbon-limiting conditions and a 20% dissolved oxygen saturation in the culture medium. A ratio of 1:3 between the yeast-extract and glycerol was considered to insure the strain necessities (considering the results from the exponential feeding cultivation of E. *coli/*pMS*ift*OptR strain). The feeding solution containing 5 g/L yeast-extract and 15 g/l glycerol was pumped into the reactor after 14 hours of fermentation when the increase in dissolved oxygen concentration indicated the carbon substrate depletion from the fermentation broth. The continuous feeding profile adopted for this experiment seemed to meet the strains metabolization capacity, since the glycerol concentration in the culture medium remained under the detection limit during the whole fermentation process. The optical density, dry cell weight and enzyme activity were monitored during the 36 hours of fermentation and the values recorded are represented in figure 4.10 as time functions.

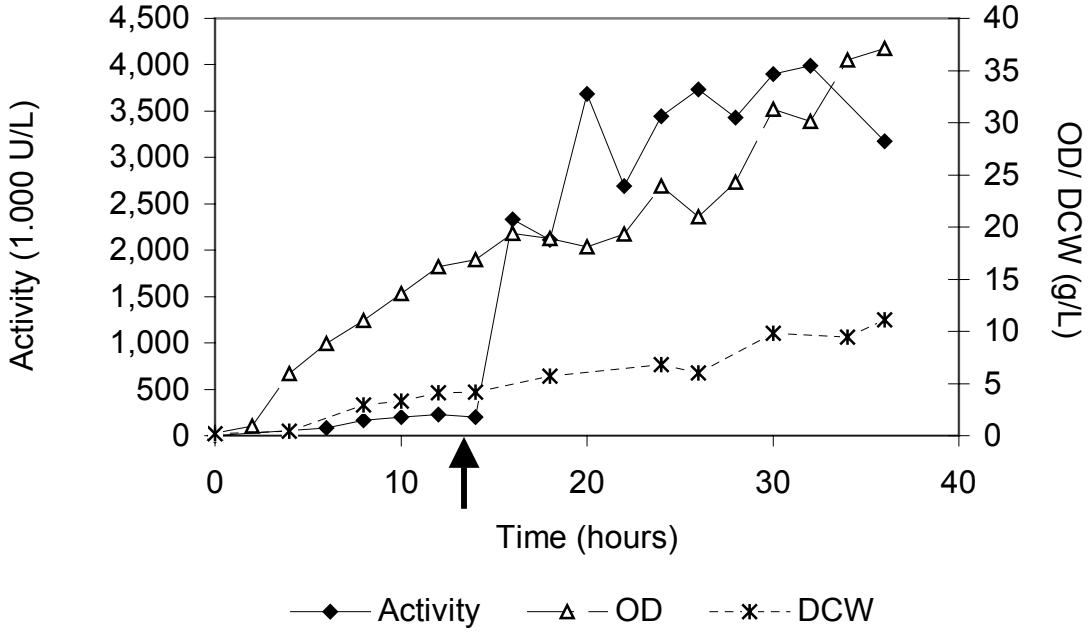


Figure 4.10. The time-courses of optical density, enzyme activity and DCW for 20 L bioreactor mass-culture medium fermentation experiment (*E.coli/*pMS*ift*OptWT strain, T=37°C, 20% oxygen saturation). At the arrow, the fed-batch process started.

A good correlation was detected between the optical density variation and the dry cell weight (see figure 4.10). In the batch phase, the cells developed exponentially after a very short lag phase of 2 hours. After the beginning of the fed-batch phase, the OD remained constant around 8 hours, time in which a metabolic switch possibly granted the capacity to adapt to the new medium conditions. Further, the culture continued to develop, the optical density and the dry cell weight reaching values of 35 and 12 g/L respectively, through the end of experiment. A rising tendency was surprisingly detected also for the enzyme activity. Some deviations between the values obtained as a result of activity test variation were recorded, but no significant loss of activity occurred: to the end of fermentation values around 3,500,000 U/L could be obtained. To protect the enzyme from the possible actions of denaturating *E.coli* proteases, the fermenter temperature was raised after 36 hours of fermentation to 60°C for 30 minutes. The cells were further isolated from the culture medium by centrifugation and to obtain the stock enzyme solution, cell disruption was performed using the high-pressure homogenizer (see 8.6.3.).

A solution with 240,000 U/L activity were obtained as stock enzyme solution as a result of this experiment, so the heating procedure led in this case obviously to a loss of activity.

4.6. Discussion

The fermentation experiments performed in the frame of this work employed media containing yeast-extract, glycerol, mineral elements and vitamins, all meant to insure a proper culture development. The feeding solutions also contained yeast extract and glycerol and the different fed-batch strategies adopted were meant to ensure C-limiting conditions and low acetate concentrations. Generally growth conditions that restrict growth of the host strain, result in increased production of the recombinant protein *(Lee and Parulekar, 1993).*

The acetate formation was controlled using glycerol as carbon source. Some authors sustain that acetate is not produced when glycerol is used as C-source but high market price (compared to glucose) make the use of glycerol prohibitive for some processes *(Yee and Branch, 1992).* On the other hand the acetate formation was reported during HCDC of *E.coli* TG1 even using glycerol as carbon source *(Korz et al., 1995)*.

Various fermentation techniques have been investigated for growing recombinant *E.coli* strains (*E. coli/*pMS*ift*OptR and *E. coli/*pMS*ift*OptWT) in batch and fed-batch cultures up to 20 L bioreactors scale. Since the goal of the project was to obtain high enzyme activities for recombinant inulase II, most of the fermentation experiments were performed of *E. coli/*pMS*ift*OptR which was reported to provide 435,000 U/L enzyme activity, a 35% increase as compared to the *E. coli/*pMS*ift*OptWT strain (320,000 U/L) when cultivated in 5 ml LB medium *(Walter et al., 2001)*. This value could not be reached when cultivation experiments were performed in similar conditions in 5 and 500 ml cultures. Even when cultivation was performed in a 1 L bioreactor and so a better aeration could be provided no higher values than 300,000 U/L could be obtained (see figure 4.2). A possible explanation was sought at the expression level of gene coding for Inulase II *(ift)*. The *ift* gene expression is under a lac-promotor control and so IPTG and lactose were used to raise the enzyme activity by increasing the gene expression. Since no significant changes in the enzyme activity could be

detected even after induction, it is assumed that the multi-copy pUC19 plasmid (which contains the *ift* gene) is constitutively expressed without necessity of induction. All cultivations were performed in the presence of ampicillin using a ratio 1:1000 between the ampicillin solution (60 µg/ml) and the volume of the culture medium. The amount of ampicillin is considered to be sufficient for an effective strain selection. *E.coli* X90strain was cultivated using even lower ampicillin concentrations (50 µg/ml) for the plasmid-containing cell selection *(Yee and Blanch, 1993)*. On the other hand, some authors reported that 100 mg/l of ampicillin added to high density cultures were degraded in less than 30 minutes by the secreted β-lactamase in the medium. *(Jung et al., 1988)*.

*E. coli/*pMS*ift*OptR strain was assayed for fermentation experiments using a yeast-extract/glycerol medium. As a first investigation step, the experiment was carried out in parallel in identical conditions in three bioreactors. In repeated experiments it was shown that cultures often developed differently even if inoculated from the same preculture. The batch phase was alike for all cultures but after the addition of feeding solution two cultures continued to develop while the growth curve for the third culture entered the stationary phase (see figure 4.3). As a result of the feeding profile the OD doublet itself compared to the third culture (see figure 4.3.). Glycerol accumulated in this culture and its consumption began only towards the end of experiment (see figure 4.4, reactor III). The high concentrations of glycerol seemed not to affect the enzyme production since 30% higher values were obtained to the end of experiment for this culture (250,000 U/L) compared with the other two cultures (150,000 U/L). Comparing cultures I and II, the small differences recorded at the growth rate levels in the exponential phases seemed to have a significant influence on the enzyme production with a difference of 50% at 20 hours of fermentation. Though, to the end of the experiment, when the stationary phase is reached the difference is minimized, activities around 150,000 U/l were achieved for both cultures.

A correlation between growth rate and recombinant product yield was observed for instance in the cellular level of human interferon alpha 1 in *E. coli*, which was

higher at low growth rates than at high growth rates, in C-limited chemostat studies *(Riesenberg, 1990)*. On the other hand, an opposite relation was observed for the production of alpha consensus interferon (IFN-αCon): the production yield increased with the specific growth rate *(Curless et al., 1990*). It seems that the influence of the growth rate on recombinant protein production has to be determined empirically. For *E. coli/*pMS*ift*OptR a better enzyme production was observed under limiting conditions (without feeding). However with this strategy the envisaged high-cell densities are not possible, therefore a different more restricted feeding seemed a good choice for further experiments.

Yeast-extract / glycerol medium was employed for an *E. coli/*pMS*ift*OptR fermentation experiment (20 l bioreactor scale). The fed-batch process had as control parameter the glycerol concentration in the culture medium and the feeding solutions contained yeast-extract and glycerol. The continuous feeding was meant to ensure C-limiting conditions that will lead to good enzyme production and prevent acetate formation, but could only proceed for one hour, since the feeding rate exceeded the culture uptake capacity. During this period, the culture exponential growth continued at higher values that in the previous batch cultivation phase (see figure 4.5). A good correlation was detected between optical density and dry cell weight. Although two further punctual feeding steps were performed no further increase of these parameters could be obtained. Towards the end of the experiment, values around 25 and 8,5 g/L were detected for optical density and dry cell weight, respectively. As a consequence of the continuous feeding profile, the enzyme production increased to 500,000 U/L. The acetate concentration was maintained below the detection limit during the whole fermentation course and the rising trend of enzyme activity is maintained even after the punctual feeding steps, but at lower rates. At the end of experiment 750,000 U/L were obtained. Jahnz employed the same strain for a similar fermentation experiment, the feeding profile being controlled in this case by the dissolved oxygen saturation in the culture medium *(Jahnz, 2001)*. The fed-batch cultivation was continuously performed even if high glycerol and acetate

concentration accumulated in the medium. At the end of cultivation an Inulase II activity of 1,700,000 U/L and 10,5 g/L dry cell weight were obtained *(Jahnz, 2001)*. This experiment was allowed to proceed only 12 hours since the high amounts of antifoam agent indicated a premature cell lyses. Even if the cultivation was not performed in C-limiting conditions, the enzyme activity is 56% higher compared to the experiment performed in the frame of this work, though only slightly differences in the dry cell weight values were obtained. It was not possible in this work to reach these high enzyme activities on Yeast-extract / glycerol medium even with a similar feeding strategy, which may be due to the different fermentation equipment. Therefore other media suitable for HCDC were tested for recombinant inulase II production.

A different feeding approach was adopted for mineral / yeast-extract medium fermentation. The fed-batch strategy was based on the dissolved oxygen content of the medium. Dissolved oxygen rapidly increases as the carbon concentration in the fermentation broth approaches zero, being a good indicator of substrate depletion *(Yee, 1992)*. In addition, a low flow speed for the feeding solution was chosen to maintain C-limiting conditions. Glycerol was the only C-source present in the feeding solution. No yeast-extract was used in the fed-batch phase because the control of the growth rate can be difficult when alternate carbon sources are supplied in fermenter *(Zabriskie et al., 1987).*

The culture developed exponentially both in the batch and the fed-batch phases but with a lower growth rate (see figure 4.6). A medium acidification occurred after 20 hours of fermentation, which is rather unusual considering the previous experiments and could be an indicator of cell lysis. The base consumption for pH control is accompanied by an increased antifoam agent demand, offering a supplementary reason that sustains the early cellular lyses hypothesis. An unusual dry cell weight of ~ 50 g/L was obtained at the end of the experiment. This may mean that DCW could have been significantly influenced by the presence of antifoamer, so that dead cells and antifoam aggregated. Moreover the unusual optical density profile (a sharp increase to ~ 100 between 20 and 32

hours of fermentation and a decrease to ~30 at the end of experiment) could be a result of the medium acidification:

- if the high values recorded between 20 and 30 hours are values quantifying indeed the cellular growth, the OD decrease could be explained by a rapid cellular lysis
- if the values recorded between 20 and 30 hours are mainly due to the amount of antifoaming agent added in fermenter leading to increased turbidity, the OD decrease could only be a return to normal status when aggregates are sedimenting or dissolving

Therefore, the exceptionally high values obtained for optical density and dry cell weight (100 OD_{600} and 50 g/L respectively) are put under question-mark. High enzyme activities were obtained, around 3,000,000 U/L but a clear trend of the activity variation could not be stated and even the enzyme activity values could have been influenced by the unusual culture development. The high intracellular activity and low free enzyme activity in the culture medium (around 80,000 U/L), on the other hand, do not sustain the cell lyses theory. However, the experiment course and the results obtained could not be reproduced in a second experiment. To the end of fermentation course significant activity lost occurred. Due to tight time schedule this mineral / yeast-extract medium was not further investigated, since another HCDF suitable medium should be evaluated for cultivation of *E. coli/*pMS*ift*OptR*.*

This so-called mass-culture medium contains yeast-extract and glycerol as carbon and nitrogen sources along with salts and trace elements *(Riesenberg et al., 1991)*. When complex carbon-nitrogen substrates (yeast-extract), are used together with carbohydrate substrates (in this case glycerol), the dissolved oxygen change is not so obvious when the carbon source is depleted, as the cells continue to utilize the complex substrates *(Suzuki et al., 1990)*. Consequently the control of the feeding profile had to take into consideration both the dissolved oxygen concentration and the glycerol depletion from the culture medium.

As a first step, the influence of the feeding procedure on the culture development and enzyme production was investigated and compared with the parameters obtained for batch cultivation. Three parallel 1 L bioreactors were inoculated with *E. coli/*pMS*ift*OptR inoculum and the fed-batch procedure was performed for only 2 out of the 3 bioreactors. The volume of the reactor permitted only a punctual feeding procedure and close to the injection point, cells were exposed to very high concentration of nutrients, although for a short time. At the same time, there are possibly also local volume elements in a reactor where the cells are starved for substrate since the consumption rate exceeded the local mass transfer *(Neubauer, 1995)*. This may cause stress to the cells, which is reflected at optical density level since all three cultures developed identically during the experiment, independent of the type of cultivation: batch or fed-batch. Immediately after the first feeding step a peak was observed for the enzyme activity (2,000,000 U/L) in the fed-batch cultures, but at the end of the experiment similar values around 1,000,000 U/L were recorded for all three cultures. The loss of activity detected for the fed-batch cultures was considered to be due to cell lysis, but no significant free enzyme amounts were detected in the culture broth: the values around 80,000 U/L do not compensate for the lack of 1,000,000 U/L inulase activity. However the enzyme could be present as inactive aggregates (inclusion bodies) in the cell debris. One possible reason for this loss of activity could be the formation of acetate and other metabolic side products, which inhibit further growth and enzyme production. The small reactor volume employed here made it impossible to carry out a slow and nutrient-limiting feeding, therefore a continuous feeding strategy was examined in a large (20 L) fermenter.

Scaling-up, continuous and exponential feeding strategies were investigated for the mass-culture medium in a 20 L bioreactor. Both methods were meant to minimize the by-product acetate formation. The feeding solutions contained glycerol and yeast-extract.

For the continuous feeding experiment, the fed-batch procedure was set to follow the glycerol depletion from the culture medium and to maintain a value around

20% for oxygen saturation in the culture broth during the fermentation experiment. The lag phase for this cultivation was longer than expected, the exponential growth started after 10 hours and consequently the glycerol was not completely metabolized when the fed-batch procedure started. For 6 hours, as a consequence of the premature start of the feeding, the glycerol concentration remained at high levels (around 20 g/L) exceeding the culture uptake capacity (see figure 4.8). The cell development continued at maximal growth rate since no carbon limitation was achieved, but no inhibitory amounts of acetate were detected in the culture medium. The highest values recorded (around 4 g/L) being below the inhibition limit for *E.coli*, which lays around 10 g/L *(Pan et al., 1987, Riesenberg, 1991.1).* In addition, no inhibition could be detected at the optical density level, which developed a constant rate. The profile of the dry cell weight variation curve recorded during this experiment matches with the optical density variation until 16 hours of fermentation. In the following period, the exponential growth certified by the optical density variation continues, though constant values are recorded for the DCW. The correlation between the optical density and the dry cell weight of the culture may change during the production of proteins that aggregate and form inclusion bodies *(Hwang and Feldberg, 1990)*. The possibility of a formation of inclusion bodies will be discussed below. To the end of cultivation, 15 g/L dry cell weight were obtained, corresponding to an optical density around 40.

The highest enzyme activity (~ 1,100,000 U/L) was recorded after 12 hours of fermentation directly before the initiation of the fed-batch procedure. After this moment, even though the culture continued to develop, a sharp decrease of the enzyme activity was recorded when the glycerol concentration in the medium exceeded the strain necessities. The decrease of volume activity continued, but at a reduced rate, even in the latest stage of experiment, when glycerol was efficiently metabolized. To the end of fermentation, an overall decrease of 70% was detected, meaning that only an activity of 310,000 U/L could be recovered from the fermentation broth. Again, no high amounts of enzyme could be identified in the culture broth as free enzyme due to cell lysis.

No improvement compared with the 1 L bioreactor experiment with identical medium was achieved. Even if a high an activity of 1,100,000 U/L were obtained after 12 hours, only 310,000 U/L could be recovered at the end of the experiment. The fed-batch process was not performed as planed due to a retarded culture development and so a dry cell weight of only 15 g/L was reached.

The cultivation in mass-culture medium led to high activities, but again a loss of activity occurred which may be due to an over-supply of nutrients in an important phase of culture development. Since high-cell densities will only be reached with feeding, a totally different strategy was employed: exponential feeding.
The exponential feeding is considered to be the most successful fed-batch strategy allowing the cells to develop at a constant growth rate below the critical rate at which acetate if formed, by limiting the carbon source concentration and the excess of dissolved oxygen in the culture broth *(Strandberg, 1991).*
The yeast-extract and glycerol concentrations in the mass-culture medium recipe were modified, starting the cultivation with 2 g/L yeast-extract and 6 g/L glycerol (ratio 1:3, instead of 1:4 for previous experiments). This medium composition was considered to insure the strain necessities for nitrogen and carbon sources in the batch phase *(Ross, personal communication).* The feeding solution was pumped into the fermenter at an appropriate flow rate to obtain a reduced growth rate for *E. coli/*pMS*ift*OptR (μ=0,12) compared with the one calculated from previous experiments under limited conditions (μ=0,25). Additionally, a 20% dissolved oxygen saturation was maintained in the cultivation medium during the course of fermentation. The fed-batch procedure was initiated as a response to a drop in dissolved oxygen concentration below 20%. The culture was able to efficiently metabolize the nutrients that were supplied with the feeding solution for 2 hours, a fact confirmed by the absence of glycerol from the culture medium. After that time the glycerol-feeding rate exceeded the strain necessities and glycerol started to accumulate in the medium, although the desired reduced growth rate is maintained and acetate concentration in the culture medium is not

significant, far from the inhibition limit. The optical density increase was mirrored at dry cell weight level (around 10 g/L were obtained through the end of experiment), but not at enzyme activity level. After a high value recorded immediately after the fed-batch procedure started (~ 2,000,000 U/L), a rapid loss of inulase activity occurred, in less than 8 hours a decrease of 50 % being recorded. For the last 15 hours of cultivation the downwards tendency is maintained but at lower rates, in the end of experiment only 550,000 U/L volume activity could be obtained. Investigating the presence of recombinant inulase II as free enzyme in the culture medium as a result of cell lyses, 80,000 U/L could be detected. This value covers only 5% from the lacking enzyme activity.

Adopting the exponential feeding profile, a lower growth rate could be induced compared to the one achieved with continuous feeding profile experiments. However the exponential glycerol dosage worked only for 2 hours an envisaged. Then obviously a switch in *E. coli* metabolism occurred, which led to an accumulation of glycerol in the fermentation broth, since exponential growth slowed down. At the same time a sharp decrease in enzyme activity could be observed. Again, the loss of activity could not be circumvented and also no high cell densities were obtained. Further experiments are needed to determine which factor triggers this switch in metabolism, leading to a reduced growth rate and a significant loss of recombinant enzyme.

One possibility explanation why inulase activity is decreasing so fast is the action of *E. coli* proteases becoming active due to cell lysis. This lysis may be caused by growth conditions (over-supply or lack of nutrients). As mentioned above the reasons for this switch are not clear at the moment, however, the proteolytic degradation of the enzyme can be inhibited by heat treatment. To prevent the actions of intracellular E.coli proteases, the bioreactors were heated at 60°C for 30 minutes. This temperature increase is supposed to destroy the proteases but has no influence over the recombinant inulase II, which is stable up to 65°C.

This procedure was carried out for fermentation of both *E. coli/*pMS*ift*OptR and *E. coli/*pMS*ift*OptWT strains, in order to obtain high activity stock recombinant enzyme solutions.

For *E. coli/*pMS*ift*OptR strain, considering the previous experiments the cultivation was stopped after 12 hours to prevent the loss of activity, the proteases that could have possible destroyed the enzyme were inactivated heating the culture broth at 60°C for 30 minutes, while the recombinant inulase II is thermostable. Investigating the enzyme activity no enzyme denaturation could be identified as a consequence of temperature increase. As a result of this experiment enzyme solution with an activity of 1,500,000 U/L was obtained.

The E*. coli/*pMS*ift*OptWT strain was also assayed for cultivation in mass culture medium. The fermentation procedure was similar as the one for the continuous fed-batch fermentation of E*. coli/*pMS*ift*OptR in mass-culture medium. The continuous feeding profile was set to maintain carbon-limiting conditions and a 20% dissolved oxygen saturation in the culture medium. A ratio of 1:3 between the yeast-extract and glycerol was considered to insure the strain necessities (considering the results from the exponential feeding cultivation of E*. coli/*pMS*ift*OptR strain). The fed-batch was initiated at a dissolved oxygen concentration increase, which indicated the carbon substrate depletion from the fermentation broth. The continuous feeding profile adopted for this experiment seemed to meet the strains metabolization capacity, the glycerol concentration in the culture medium remaining under the detection limit during the whole fermentation process.

A good correlation was detected between the optical density variation and the dry cell weight (see figure 4.10). In the batch phase, the cells developed exponentially after a very short lag phase (only 2 hours). After the beginning of the fed-batch phase, the growth rate remained constant for 8 hours. The culture continued to develop, the optical density and the dry cell weight reaching values of 35 and 12 g/L respectively, at the end of the experiment. An increasing tendency was surprisingly detected also for the enzyme activity. Some deviations

between the values obtained as a result of activity test were recorded, but no significant loss of activity occurred: to the end of fermentation values around 3,500,000 U/L could be obtained. Again the fermenter was heated (60°C) as a precaution against proteases. Surprisingly this time the heat treatment led to loss of enzyme activity, only 240,000U/L could be obtained as stock enzyme solution. This may be due to protein aggregates formed between *E. coli* cells and inulase.

The loss of activity while cultivating recombinant *E. coli* strain could be observed in later fermentation stages (~ 12 hours), it does not seem to be a consequence of the medium or the fed-batch strategy, since it occurred under differing conditions. There are several possibilities for this loss: the enzyme could be destroyed by proteases derived from external sources. This does not apply to the fermenter but for the process of cell harvest, disruption and activity test. To exclude this possible contamination with external proteases the culture broth samples were handled in parallel under unsterile and sterile conditions until the enzyme solution was assayed for activity test. No evidence of external proteases influence could be demonstrated.
Therefore it must be assumed that there is a real loss of active protein during cultivation. This could be due to E. coli proteases that became active due to culture conditions as mentioned above. Heat treatment of fermentaion broth was carried out as a precaution and led to good results for *E. coli/*pMS*ift*OptR cultivation. However the same treatment could not prevent a loss of activity in the case of *E. coli/*pMS*ift*OptWT fermentation, which makes the action of proteases less probable. Moreover, culture samples were taken before and after the loss of activity during *E. coli/*pMS*ift*OptR fermentation and assayed for SDS-electrophoresis. They led to identical protein patterns (data not shown), suggesting that no proteolytic cleavage of inulase had occurred which should have led to a variety of smaller protein fragments.

Another possibility which would lead to a loss of activity is the formation of so-called inclusions bodies. This is a phenomenon often encountered in

recombinant *E. coli* protein production, where high amounts of extrinsic protein are accumulated inside the cells *(Riesenberg, 1991, 1)*. At a certain point, the solubility is not sufficient any longer and the protein aggregate and form inclusion bodies, which cannot be detected by activity test. The profiles of activity observed in fermentation of *E. coli/*pMS*ift*OptR would fit well into this hypothesis, where a rapid increase in activity can be observed, meaning a high amount of protein in the cells. The decline occurs in 1-2 hours, which could be explained by the aggregate formation. However the further slow decline of activity does not fit quite well into this theory. Also the examination of cultures samples by SDS-gel electrophoresis did not lead to unambiguous results.

The formation of inclusion bodies can be often circumvented by cultivating the strain at lower temperatures (for instance at 27°C) *(Ohta et al., 1993, Korz et al., 1995).* This leads to a slowing- down of growth and metabolic processes and often effectively prevents the forming of inactive aggregates. Therefore such an experiment has to be carried out, it will show if inclusion bodies are really causing the loss of inulase activity observed repeatedly during fermentation of the *E. coli/*pMS*ift*OptR strain.

Chapter 5

Immobilization

5.1. Introduction

The adsorption of an enzyme onto an insoluble support is the simplest method of enzyme immobilization. The procedure consists of mixing together the enzyme and the support material under certain conditions and then separating the two phases (soluble and insoluble). An important disadvantage of this method of immobilization is that the enzyme is not firmly bound to the support, so, to reduce the enzyme desorption, a cross-linking step is recommended (generally with glutardialdehyde) *(Nishimura et al., 1990)*.

The adsorption procedure is mainly due to multiple salt-linkages, which are affected by certain factors as the pH, the ionic strength, the temperature, etc. Even small changes in the experimental conditions can cause the desorption of the enzyme from the support as they affect the salt-linkage. Besides salt-linkages other weak binding forces are involved in the adsorption of the enzyme to a support (such as hydrogen bonds, Van der Waals forces).

The immobilization is often not economical for cheap row-enzymes since the carrier and the immobilization price will not be compensated *(Uhlig, 1991)*. Compared to free enzymes, the immobilization of enzymes gives the possibility

to use the immobilized biocatalysts either repeatedly, in batchwise reactions, or continuously, in tubular reactor systems. This repeated use leads to significantly lowered amounts of enzyme required for a certain biotransformation process. Further more, immobilized enzymes are often more stable than the free biocatalysts. Another advantage is that better and easier controlled processes can be performed and the biocatalyst can be easily recovered from the reaction mixture. These considerations were determining factors in seeking for an appropriate immobilization technique for *E.coli* Inulase II, involving a suitable resin.

The selection of an ion-exchanger resin is based on the compatibility of the matrix with the polarity of the protein active form. The denaturation of the protein (caused for instance by changes in the three-dimensional structure, ionic charge, chemisorption) during adsorption had to be avoided.

Inulase II was immobilized until now, as reported in the literature, by covalent binding on porous glass *(Baron et al., 1996)*, adsorption on bentonit or entrapping the whole cells in alginate *(Neubauer, 1998)* and entrapping the free enzyme in calcium alginate hydrogels *(Janhz et al., 2001)*. In this chapter investigations on the adsorption of Inulase II on anion-exchangers will be presented.

To obtain the enzyme solution for immobilization experiments, large-scale fermentations (20 L bioreactor) had to be performed for both strains *E.coli/*pMS*ift*OptWT and *E.co*li*/*pMS*ift*OptR (see 4.5.3.) and the cells were disrupted using ultrasonication (for the immobilization experiments in which the adsorption behaviour of some anion-exchanger resins was tested, see 3.2. and 8.6.2.) and high-pressure homogenizer (see 3.3. for the device description and 8.6.3. for the disruption method). The immobilization procedure is presented in the Material and Methods chapter (section 9).

5.2. Ion-exchangers

Anion exchangers are a rather cheap material for immobilization and they are also available in the quantities required for a technical scale process.

The immobilization of Inulase II from *Arthrobacter* sp. Bu0141 on anion exchange resins has been tested for different buffers and different pH-values *(Walter, personal communication).* The resin charged with different anions was tested (OH^-, Cl^- and PO_4^{3-}) and also the influence of equilibration buffer pH was investigated. For OH^- ions, it was difficult to adjust the pH to the desired value and so phosphate buffer solutions with pH values in the range of 6 to 9 were used for further investigations on the resin equilibration.

Inulase II from *Arthrobacter* sp. was immobilized successfully on anion exchange resins. Since the enzyme could be bound without a drastic loss of activity, a similar procedure, concerning the conditions of buffer and pH, was envisaged for recombinant inulase from *E. coli/*pMS*ift*OptR and *E. coli/*pMS*ift*OptWT. However, the wild-type inulase of *Arthrobacter* can be found in culture supernatant which contains little protein *(Neubauer, 1998)*. The recombinant inulase on the other hand is present in a mixture of *E. coli* intracellular proteins, which could well influence the yield and efficiency of immobilization, so it had to be tested anew for the resins and conditions mentioned above.

*E.coli/*pMS*ift*OptR and *E.coli/*pMS*ift*OptWT enzyme solutions, obtained as presented above had a protein content of ~ 40 g/L (determined following Bradford, see 8.5.). The enzyme solutions had activities of 4,000 U/L - *E.coli/*pMS*ift*OptR and 3,500 U/L - *E.coli/*pMS*ift*OptWT, respectively (25 % inulase II of total protein). These were investigated with respect to the adsorption behaviour with the following resins: Duolite A 561, Duolite A 568, Amberlite IRA 67, and Amberlite IRA 94 S (Fa. Rohm and Haas Deutschland GmbH, Frankfurt/

Main). All the investigated anion-exchangers are weakly basic resins and some parameters of these resins are summarized in table 5.1.

Table 5.1. Ion-exchangers basic parameters

Resin name	Structure	Particle size (µm)	Matrix type	Functional groups
Amberlite IRA 67	gel structure	500-750	acrylic-DVB*	$-N-(R)_2$
Amberlite IRA 94 S	macroporous	350-470	styrene-DVB	$-N-(R)_2$
Duolite A 561	macroporous	470-740	phenol-formaldehyde	$-N-(R)_2$
Duolite A 568	macroporous	150-600	phenol-formaldehyde	$-N-(R)_2$

* divinylbenzene

The pH range for immobilization was appropriately chosen to exclude the enzymes isoelectric point (for *Arthrobacter* sp. Inulase II = 4.5) *(Walter, personal communication)* considering that for pH values lower than the enzymes isoelectric point the cationic enzyme form is stabilized and so very poor adsorption onto an anion-exchanger resin should be obtained. A good adsorption onto the material investigated was expected for pH values higher than the isoelectric point, when the enzyme will be stabilized in overall anionic form. The activity of the immobilized enzyme increases with the buffer pH value (data not shown) and so resin equilibration with phosphate buffer, pH 9 was considered appropriate for further experiments.

3g equilibrated resin and 30 ml diluted enzyme solution were mixed and incubated 24 hours at room temperature. After washing and cross-linking with glutardialdehyde (3 g/L glutarhialdehyde concentration, the reaction was carried out at room temperature for 3 hours), around 3 g of immobilized enzyme were

obtained (see 8.9.1.2. and 8.9.1.3.). To quantify the yield of immobilization, activity tests were performed for soluble (native) enzyme and for immobilized enzyme. The activity test for native enzyme was performed according to the standard procedure (see 8.10.1.1.) and the following results were obtained: 4,000 U/L for *E.coli/*pMS*ift*OptR enzyme solution and 3,500 U/L for *E.coli/*pMS*ift*OptWT enzyme solution. Table 5.2. summarizes the immobilized activity and the immobilization yield obtained for the resins investigated. The values were calculated as percent of immobilized enzyme activity related to the free enzyme activity.

Table 5.2. Immobilization efficiency. The initial enzyme activities were 4,000 U/L for *E.coli/*pMS*ift*OptR and 3,500 U/L for *E.coli/*pMS*ift*OptWT.

Resin	***E.coli/*pMS*ift*OptR**		***E.coli/*pMS*ift*OptWT**	
	$Act_{imm.}$(U/L_{resin})	**Yield (%)**	**$Act_{imm.}$(U/L_{resin})**	**Yield (%)**
Duolite A 568	825	20.6	750	19.3
Duolite A 561	180	4.5	150	4.2
Amberlite 94 S	790	19.8	660	19.0
Amberlite IRA 67	70	1.7	56	1.6

The results presented in table 5.2. show that ~20% from the free enzyme activity was detected as immobilized activity when the enzyme was adsorpted on Duolite A 568, which corresponds to around 800 U/L_{resin}. When the immobilization was performed with Amberlite 94 S, around 750 U/L_{resin} immobilized activity were obtained for both enzyme solutions, representing an immobilization yield of ~19.5%. The values obtained for Duolite A 568 and Amberlite 94 S make those two resins more suitable for further employment than Duolite A 561 and Amberlite IRA 67 which only bound up to 5% of enzyme.

5.3. Adsorption and Cross-linking

To run an efficient adsorption it is necessary to determine the maximum loading of the enzyme to the carrier, which refers to the maximum amount of enzyme that can be immobilized on a certain amount of support *(Woodward, 1984)*. For an optimal immobilization it is important to determine the ratio of the enzyme activity prior to immobilization and the activity found after immobilization.

The stock *E.coli/*pMS*ift*OptR Inulase II solution obtained after mass culture medium fermentation (see 4.5.3) and high-pressure homogenizer cell disruption (see 8.6.3) had an activity of 1,500,000 U/L. To investigate the maximum loading of the resin, the enzyme solution was diluted with distilled water and assayed for immobilization on Duolite A 568. 5 g of equilibrated resin slurry were incubated at room temperature with 50 ml enzyme solution (pH 6.5) having different enzyme activities (volume ratio 1:10 between resin and enzyme solution). The enzyme activity was in the range of 1 to 300 U/ml (for immobilization procedure see 8.9). Duolite A 568 was further used for immobilization because in several experiments a higher activity than on Amberlite 94 S could be achieved.

Activity tests were carried out for 1 ml of immobilized enzyme and the DFAIII concentration was determined (see 8.10.1.1) and used to calculate the activity of immobilized enzyme. In order to calculate the loading of the 5 g resin slurry used for immobilization, this activity value was expressed as U pro ml resin.
In order to calculate the immobilization yield, the activity of the immobilized enzyme was related to the enzyme activity employed for immobilization.

Activity tests were also performed to determine the amount of enzyme, which remained unbound after immobilization. Before cross-linking with glutardialdehyde, the liquid phase containing the unbound enzyme was separated from the immobilized enzyme by pouring off the supernatant. 100 µl from this solution was tested for activity and the DFAIII concentrations obtained

were calculated as activity of the unbound enzyme. The variations of immobilized activity and immobilization yield are presented in figure 5.1, plotted against the initial enzyme activity (in U pro ml enzyme solution).

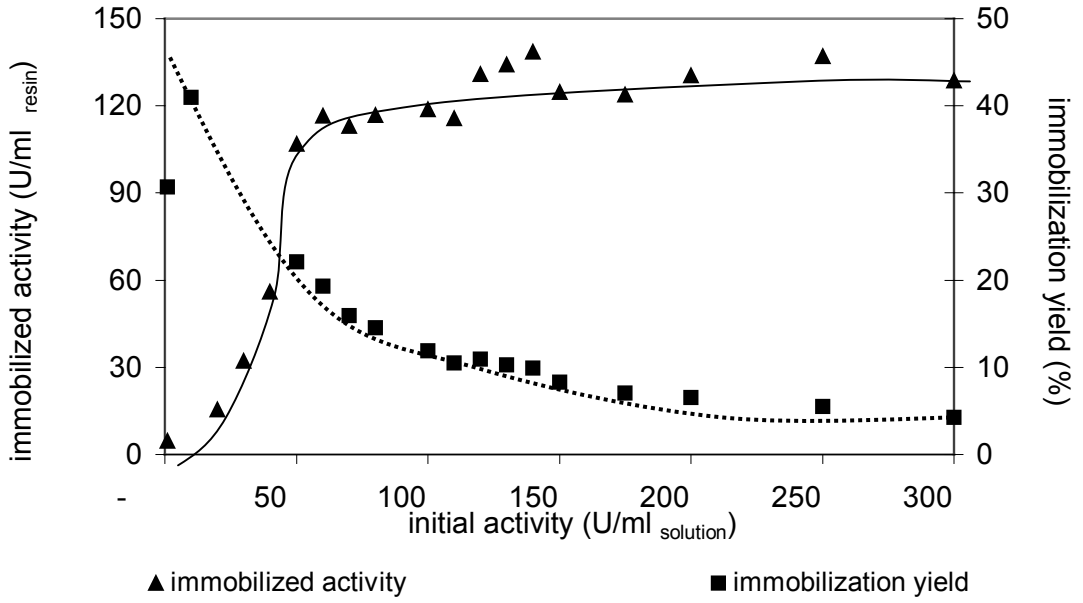


Figure 5.1. The correlated of immobilized activity and immobilization yield with the initial enzyme activity. The curves were obtained for *E.coli/*pMS*ift*OptR enzyme immobilized on Duolite A568 and a ratio 1:10 for resin and enzyme solution.

The immobilized activity increases with the initial enzyme activity in solution up to a threshold value of about 50 $U/ml_{solution}$. Beyond this value, the maximum immobilized activity steady state is obtained without significantly increasing further with higher initial activity in solution. Regarding the immobilization yield, a rapid decrease occurs with the increase of the initial activity. The highest value (almost 50 % immobilization yield) is obtained for 1 $U/ml_{solution}$ initial activity but the immobilized activity is very low (5 U/ml $_{resin}$). With a high loading of the resin the efficiency is decreasing. A well-balanced process, considering both the immobilized enzyme activity and the immobilization yield, was obtained for an

initial activity of 60 $U/ml_{solution}$ when the immobilized activity is 120 U/ml_{resin}. The ratio resin - enzyme solution being 1 to 10, the immobilization efficiency was calculated to be around 20%. Only small concentrations of DFAIII were obtained when the amount of unbound enzyme was investigated; calculating the enzyme activity, less than 1% from the total enzyme used for immobilization could be detected in solution. Even when the enzyme activities used for immobilization exceeded the maximum loading of the resin, the amount of unbound activity did not match with the excess of enzyme.

The same experiment was performed for *E.coli/*pMS*ift*OptWT enzyme. The stock enzyme solution had an activity of 240,000 U/l and was appropriately diluted with distilled water to cover the whole range of activity until 1U/ml (the pH value for the enzyme solutions used for immobilization was 6.5). Further, the experiment was conducted as described for the *E.coli/*pMS*ift*OptR enzyme. The results are shown in figure 5.2.

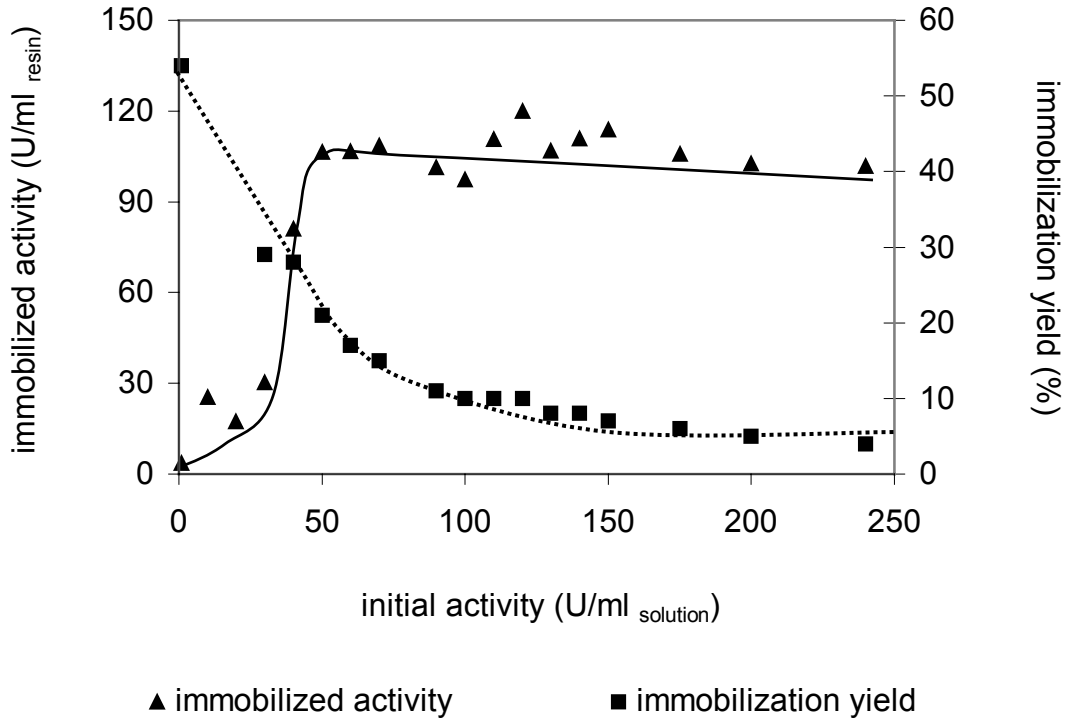


Figure 5.2. The correlated of immobilized activity and immobilization yield with the initial enzyme activity. The curves were obtained for *E.coli/*pMS*ift*OptWT enzyme immobilized on Duolite A568 and a ratio 1:10 of resin and enzyme solution.

The correlation is similar to the one for *E. coli/*pMS*ift*OptR enzyme. The immobilized enzyme activity recorded for this experiment increases with the initial enzyme activity up to 50 $U/ml_{solution}$ when the immobilized activity is 110 U/ml_{resin}. This is the maximum value for the immobilized activity and it remains constant independent of the initial enzyme activity used for immobilization. The efficiency of the immobilization process decreases very rapidly with increasing initial enzyme activity. When only 1 $U/ml_{solution}$ was used for immobilization, the immobilization efficiency is 54%, but the immobilized activity is very low. If the value for the immobilized activity remains constant around the maximum value of 110 U/ml_{resin}, it is natural that the immobilization process is less efficient with the increase of the enzyme concentration, which can be directly correlated with the

enzyme activity. A maximal resin loading is reached when the immobilization is performed with a solution having an activity around 50 $U/ml_{solution}$. The efficiency of the process was calculated to be 20% for this solution. The free enzyme activity increases with the initial concentration of the enzyme employed for immobilization, but does not fit with the excess of enzyme when the maximum loading of the resin was reached. For both enzyme solutions, up to 50% of enzyme could not be detected after immobilization. Possible reasons for this loss of activity are given in the Discussion part.

For both enzyme solutions a similar loading of anion exchange resin with ~ 120 U/ml_{resin} could be obtained.

Cross-linking with Glutardialdehyde

A bi- or multifunctional compound is generally used to insure a stable binding between enzyme and the carrier. Glutardialdehyde ($C_5H_8O_2$) is often employed as cross-linking agent and serves as reagent for intermolecular cross-linking of the biocatalyst without involving the carrier. The aldehyde groups from both ends of the glutardialdehyde molecule react with free amino groups such as ε-amino groups and N-terminal amino groups from the enzyme molecule. Several other bi- or multifunctional compounds, such as toluene diisocyanate and hexamethylene diisocyanate have also been successfully used.

$O=CH-CH_2-CH_2-CH_2-CH=O$	**$O=C=N-(CH_2)_6-N=C=O$**
($C_5H_8O_2$)	**($C_8H_{12}N_2O_2$)**
glutardialdehyde	hexamethylene diisocyanate

Compared with the simple adsorption immobilization, the result of the combined adsorption-cross-linking method is a more stable immobilization product. The stability refers to the fact that small changes in the surrounding of the

immobilized enzyme, such as pH and ionic strength, often cause the detachment of the biocatalyst from the support. The cross-linking is therefore used to fix the enzyme on the carrier surface. On the other hand, some authors refer to the cross-linking as having the disadvantage that only relative small amounts of enzyme, and low activity, respectively, per carrier will be linked since only the surface of the enzyme-carrier particle is involved in the cross-linking reaction *(Tanaka, 1999)*.

A critical parameter of the immobilization procedure is the concentration of cross-linking agent (glutardialdehyde). It can be expected that glutardialdehyde will cause a decrease of activity, either affecting the three-dimensional structure of the biocatalyst or aggravating the substrate diffusion difficulties, limiting the access to the catalytic center. The influence of the cross-linking with glutardialdehyde on the immobilization efficiency was largely investigated by Monsan and co-workers *(Monsan et al., 1977/78)*.

To establish also an immobilization procedure for Inulase II from *Arthrobacter* sp. BuO141 different concentrations of glutardialdehyde were tested. *(Walter, 2000)*. When the enzyme was immobilized by adsorption on anion-exchangers, the cross-linking with 5 g/l was found to be effective for a stable immobilization. No enzyme deactivation was recorded when the immobilized enzyme was repeatedly employed for inulin-juice transformation and glutardialdehyde concentrations up to 30 g/L showed to have no influence over the enzyme activity *(Walter, 2000)*. For an efficient cross-linking process, even a lower glutardialdehyde concentration (3 g/L) was considered adequate *(Walter, personal communication)*. Since the recombinant inulase is (nearly) identical, for the immobilization of Inulase II from *E. coli*/pMS*ift*OptR and *E. coli*/pMS*ift*OptWT on anion-exchangers resins, the cross-linking was also performed with 3 g/L glutardialdehyde. The reaction was allowed to proceed for 3 hours at room temperature under low speed rotation, to insure a better distribution for the glutardialdehyde solution. The supernatant was poured off and the cross-linked

immobilized enzyme was obtained and further assayed in activity tests. This glutardialdehyde concentration was shown to be effective for a stable immobilized biocatalyst and to have no influence over the enzymatic function. This procedure therefore was considered to be appropriate for a stable immobilized product when assayed for the repeatedly employment and the continuous biotransformation of inulin.

5.4 Repeated Biotransformation of Inulin

The performances of biocatalytic process are quantified, among other parameters, by the operational stability expressed as catalytic half-life, ($\tau_{1/2}$), defined as the period of time in which the catalytic activity of the enzyme decreases to 50% of its initial value. It is to be expected that the catalytic activity of the enzyme will decline over time under the reaction conditions, still in order to obtain reliable information, extrapolation procedures have to be avoided *(Buchholz, 1987)*.

The continuous batchwise biotransformation of inulin was realized using immobilized *E. coli/*pMS*ift*OptR enzyme in a tubular reaction system. The immobilization – according to the standard procedure (see 8.9) - was carried out with 60,000 $U/L_{solution}$ (to insure the maximum loading of the resin) and the immobilized activity obtained was 130,000 U/L_{resin}. 6 g immobilized enzyme were employed for the repeated batchwise biotransformation of 1,41 L Cosucra inulin solution at pH 5.25 and 60°C, containing 150 g/L inulin. The experiment is described in chapter 8.10.1.4.

The inulin concentration in the batch solution was chosen to be higher than the maximum amount that can be catalyzed in 24 hours. If smaller concentrations would have been used, the decrease in the catalytic activity would have been

masked since the entire catalytic capacity of the immobilized enzyme would not have been exploited (incomplete conversion).

The DFAIII concentration was measured every 24 hours in the batch inulin solution and the biotransformation yield was calculated. The experiment was allowed to proceed for a sufficient period of time, long enough to permit the determination of $\tau_{1/2}$ without referring to extrapolation methods.

In figure 6.9. the variation of the biotransformation yield in time is presented.

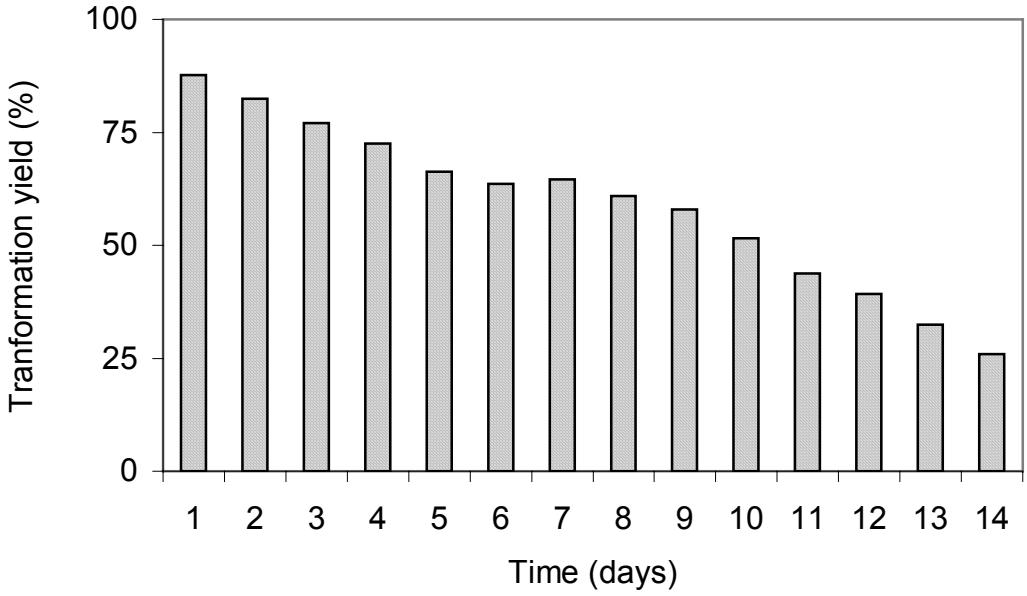


Figure 6.9. Repeated biotranformation of inulin. (*E. coli/*pMS*ift*OptR enzyme 130,000 U/L_{resin} immobilized activity, 1.41 L inulin solution containing 150 g/L Cosucra inulin, phosphate buffer pH 5.25, T=60°C, 24 hours batchwise process)

The biotransformation yield was calculated assuming the fact that, in the case of a total conversion, 60 g/L DFAIII can be obtained from 100 g/L inulin solution. As it can be seen in figure 6.9, the transformation yield decreases almost constantly and reaches 50% of its initial value after 11 days. Considering these results, a half-life of 11 days results for immobilized *E. coli/*pMS*ift*OptR inulase.

5.5. Discussion

In order to characterize the immobilization of an enzyme by adsorption onto a carrier, a considerable number of parameters are required. The carrier selection and the range of suitable activities (acceptable effectiveness) are esential for the adsorption process *(Buchholz, 1987)*.

The resins investigated for the immobilization of *E. coli* Inulase II are weakly basic anion-exchangers, having an acryl-DVB matrix type for Amberlite IRA 67, styrene DVB for Amberlite 94 S or phenol-formaldehyde for Duolite resins and tertiary amine as functional groups for all four resins examined. Calculating the activity of the immobilized enzyme, 2 out the 4 tested resins proved to bind Inulase II from *E. coli/*pMS*ift*OptR and from *E. coli/*pMS*ift*OptWT with acceptable efficiency. The results are comparable for both enzymes: the efficiency of the immobilization on Duolite A 568 is around 20% and for Amberlite 94 S around 19,5%. The other 2 resins, Duolite A 561 and Amberlite IRA 67, showed only a poor efficiency, 4.2% and 1.7 %, respectively, therefore it can be stated that they are not suitable for *E.coli* Inulase II immobilization and were no longer employed.

Adsorption experiments were performed with Duolite A 568 in order to establish the maximum loading of the resin. Using a wide range of enzyme concentrations, the maximum immobilized activity was found to be around 110 U/ml_{resin}. The immobilized activity increases up to an activity of 50 $U/ml_{solution}$ for the enzyme solution used for immobilization and remains constant at higher concentrations. The maximum immobilized activity is similar for both enzymes investigated (*E. coli/*pMS*ift*OptR and *E. coli/*pMS*ift*OptWT) which is not surprising considering that the only difference between the two enzymes at amino-acid level (primary structure) consists in the replacement of one glycine residue by arginine. Entrapping the *E. coli/*pMS*ift*OptRM inulase II in calcium alginate hydrogels, the immobilized activity reached was of 196 U/g *(Jahnz, 2000)*. For the cell-bound inulase II from *Arthrobacter ureafaciens* ATCC 21124 entrapped in calcium

alginate, only an immobilized activity of 77 U/g was achieved *(Neubauer, 1998)*. Considering these results the adsorption on anion exchangers seems to be a more suitable immobilization method for inulase II.
The immobilization yield was calculated considering the ratio of the initial and the immobilized enzyme activity. Around 50% from the initial activity was found as immobilized activity for very low enzyme loading as can been seen from figures 5.1 and 5.2. A rapid decrease in the activity yield was observed with increasing enzyme loading, while the immobilized activity remained constant at around 110 U/ml_{resin}. This lack of immobilized activity may be due to the crowding of other proteins on the support with a direct effect on the accessibility of enzyme molecules to the adsorption material *(Woodward, 1984)*. The recombinant Inulase II from *E. coli/*pMS*ift*OptR and *E. coli/*pMS*ift*OptWT being an intracellular enzyme it is necessary to disrupt the cells in order to release it. No enzyme purification was performed before immobilization, so the enzyme molecules are competing with all the other host cellular proteins for the active functional groups of the resin.

On the other hand, when the immobilized activity remains constant with the increase of the enzyme loading on the support, one could expect to find an increasing amount of unbound enzyme. Still, the activity detected for the free enzyme does not fit an activity balance. For instance, when the immobilization was carried out with 240 $U/ml_{solution}$, the immobilized enzyme activity was 110 U/ml_{resin} and the immobilization yield was 5%. That means that only 5% enzyme bound to the resin and only 12% (instead of 95 %) could be detected as free enzyme (unbound) after immobilization. This important difference could possibly be explained by the fact that the enzyme molecules create overlapping layers surrounding the carrier particle. By standard activity tests, the exact quantification of the amount of enzyme involved in this immobilization process is not possible since, due to steric impediments, only the upper immobilized enzyme strata may participate in the enzymatic reaction.

In order to avoid the desorption under conditions to which the enzyme is subjected under reaction conditions (for instance pH, temperature, ionic strength), a subsequent immobilization step (cross-linking with glutardialdehyde) was performed. A thorough investigation on the cross-linking with glutardialdehyde was carried out by Monsan and co-workers *(Monsan, 1977/78)*. Investigating the influence of glutardialdehyde over the glucoamylase activity, Nishimura *et al*. found that up to ~5.5%, the concentration of the cross-linking agent has no influence on the activity of the immobilized enzyme *(Nishimura et al., 1990)*. The immobilization was realized by covalent cross-linking of the enzyme with glutardialdehyde to an amino-group containing carrier (aminated silica gel) in the presence of a phenolic carboxylic acid (tanic acid) and a basic polysaccharide (chitosan). When the immobilization was made in the presence of glutardialdehyde, the enzyme activity was higher than the one obtained without cross-linking. The treatment with glutardialdehyde showed a 2-fold improvement in the stability of immobilized enzyme when the continuous saccharification of dextrin was performed, along with a 3-4-fold improvement in the heat stability. The Inulase of *Kluyveromyces fragilis* can be immobilized in the cells *(Workman, 1984)* by glutardialdehyde treatment. The behaviour of the enzyme in immobilized form is similar to the soluble one. Although the exact nature of the immobilization is not fully understood, no reduction of the enzyme activity was observed after glutardialdehyde treatment and the enzyme activity was not affected by the glutardialdehyde concentration. When the enzyme was entrapped in calcium-alginate hydrogels *(Jahnz, 2001)*, a glutardialdehyde concentration of 2.5 g/l showed not to affect the activity of the immobilized enzyme. 196 U/g were immobilized using increasing glutardialdehyde concentration and a reduction to 188 U/g (representing 80% from the initial activity) was registered when glutardialdehyde concentrations higher than 5 g/l were used. For the immobilization of the *E.coli* Inulase II on anion-exchangers resins, the cross-linking with a 3 g/l glutardialdehyde solution was considered appropriate and was shown not to lead to an enzyme deactivation.

The half-life of immobilized *E.coli/*pMS*ift*OptR enzyme solution was established investigating the behaviour of the immobilized biocatalyst for the repeated biotransformation of inulin. The decay of the catalytic activity could be masked by mass transport phenomena *(Buchholz, 1989).* In order to avoid the use of an extrapolation procedure, the experiment was carried out long enough to obtain a reliable value for $\tau_{1/2}$. A 50% decrease in the biocatalytic activity of immobilized *E.coli/*pMS*ift*OptRM enzyme solution occurred after 11 days.

Chapter 6

Enzyme Characterization

6.1 Introduction

Inulinfructotransferase (depolymerising) (E.C 2.4.1.93, Inulase II) converts inulin into DFAIII and fructooligosaccharides through intramolecular transfructosylation. It is necessary to characterize and compare the inulase II isolated from different sources since for the optimization of DFAIII production is important to use a highly active, and selective biocatalysts which is stable over long period of time *(Mozhaev, 1993)*.

In order to characterize the inulase II, different investigations were carried out. The molecular weight was estimated by SDS-gel electrophoresis and compared with the value obtained by sequencing the DNA. The influence of pH and temperature on the DFAIII production was investigated for both *E.coli* pMS*ift*OptWT and pMS*ift*OptR enzyme solutions. For optimal working conditions, the time course of DFAIII formation and the Michaelis-Menten kinetic parameters were determined. The same investigations were also performed for the immobilized enzyme.

The enzyme solutions of *E.coli/*pMS*ift*OptR and *E.coli/*pMS*ift*OptWT were obtained after performing the following steps:

- *E.coli* cultivations in 20 L fermenter with mass-culture medium (see Chapter 8, section 3.3.3.2),
- cell disruption using ultrasonication (the *E.coli/*pMS*ift*OptR cell suspension is ten times more diluted than the pMS*ift*OptWT prior to cell disruption, (see 3.2.) was used for investigations of the free enzyme; cell disruption using the high-pressure homogenizer was used for immobilized enzymes,
- immobilization: by adsorption on anion-exchangers (see 8.9.).

Unless otherwise stated, the activity test was performed according to the standard procedure described in Chapter 8 section 10.

6.2 Free Enzyme

6.2.1 Molecular Weight

The molecular weight of the *E.coli/*pMS*ift*OptWT enzyme was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12.5% gel). The procedure is described in Chapter 8 section 8 and the results obtained are presented in figure 6.1.

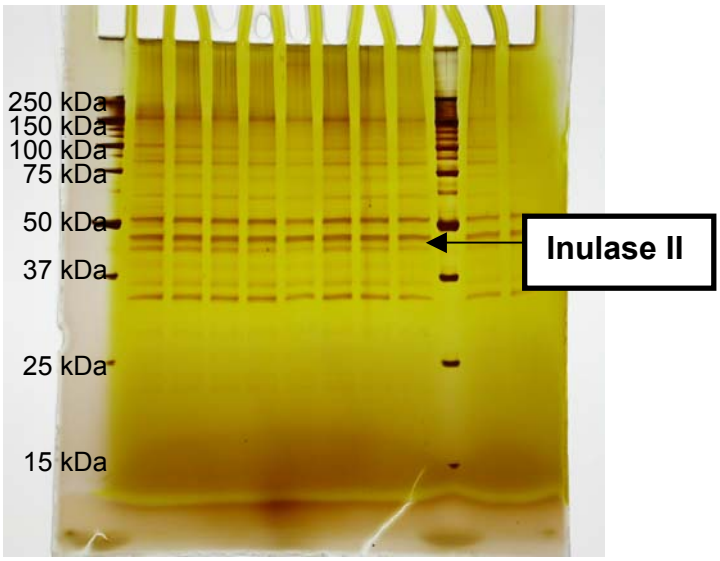


Figure 6.1. Polyacrylamide gel electrophoresis of *E.coli/*pMS*ift*OptWT enzyme. The gel was stained by silver-staining method described in Chapter 8, section 8.2. Lanes 1 and 10 = protein standard , 2 – 9= enzyme solution.

In figure 6.1. the result of the gel electrophoresis is shown. The lanes 1 and 10 correspond to the marker protein and the other ten lanes to the *E.coli/*pMS*ift*OptWT enzyme solution. The molecular weight of the Inulase II was estimated to 46 kDa which is in good accordance with the result obtained by sequencing the DNA and determining the number of amino acids of the native enzyme without signal peptide, when a molecular weight of 45.1 kDa was found *(Schubert, 1999)*.

6.2.2 Effect of pH on the Enzyme Activity

The substrate-binding site of an enzyme contains, in many cases, basic or acid groups and the overall conformation of a protein molecule is affected by the acidity or alkalinity of the solution, because the interaction of the conformation-

determining groups depends upon their ionization state. This pH dependence is very complex and there is no general equation for its description *(Ballesteros & Boross, 2000)*.

The effect of the pH on the *E.coli* Inulase II activity was measured in the range of 4.0 to 7.5. The enzyme reaction was carried out according to the standard procedure (see Chapter 9, section 10) except that the pH of inulin solution was changed. The following buffer systems were used: 0.1 M acetate buffer for pH values < 5 and 0.04 M phosphate buffer for pH values > 5. The DFAIII concentrations were measured using HPLC and can be directly correlated with the enzyme activity. In figure 6.2. the results for *E.coli/*pMS*ift*OptWT enzyme are shown and in figure 6.3. the ones for *E.coli/*pMS*ift*OptR enzyme solution.

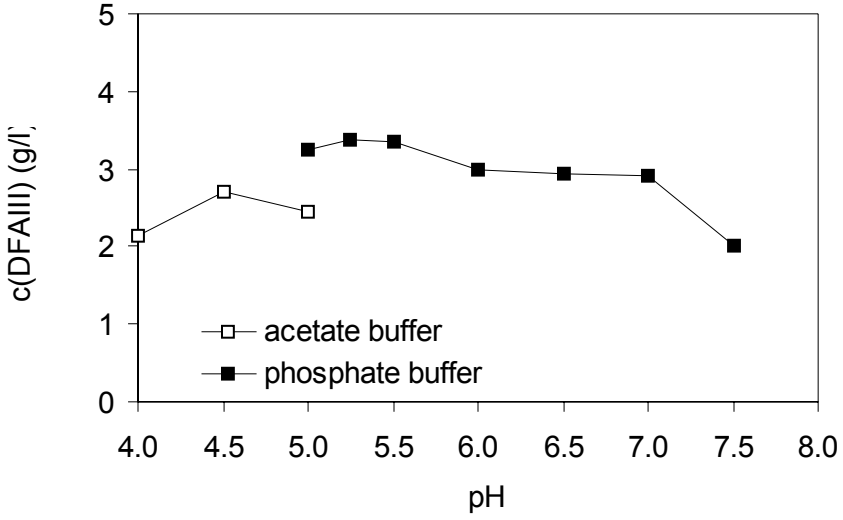


Figure 6.2. Effect of pH on the DFAIII production for *E.coli/*pMS*ift*OptWT enzyme solution (0.1 M acetate buffer, 0.04 M phosphate buffer, T= 50°C, Dahlia inulin concentration 100 g/L)

As shown in figure 6.2. the maximum DFAIII concentration was obtained at pH 5.25 (phosphate buffer). Similar results were obtained for *E.coli/*pMS*ift*OptR enzyme solution as presented in figure 6.3.

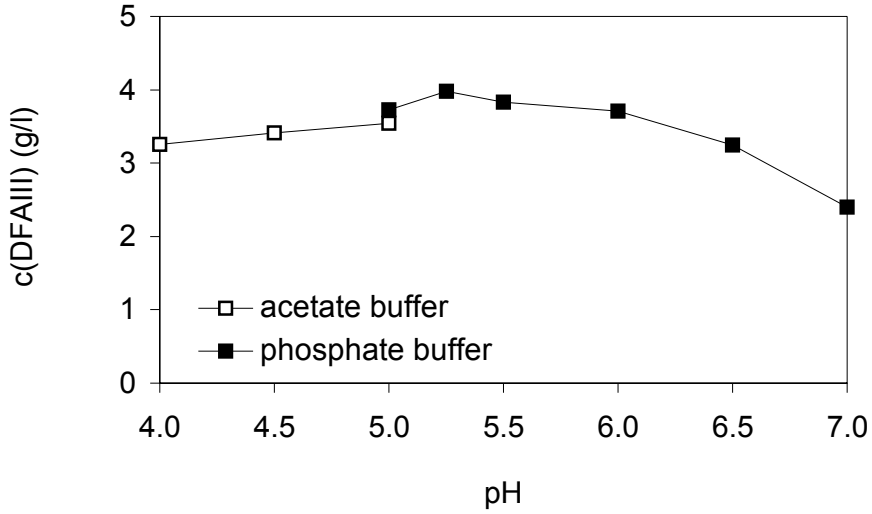


Figure 6.3. Effect of pH on the DFAIII production for *E.coli/*pMS*ift*OptR enzyme solution (0.1 M acetate buffer, 0.04 M phosphate buffer, T= 50°C, Dahlia inulin concentration 100 g/L)

The fact that at pH 5, the DFAIII concentrations obtained for acetate buffer are lower than the ones obtained for phosphate buffer, might suggest that the nature of the ion is influencing the enzyme activity. For further experiments, the optimum pH for the *E.coli* Inulase II reaction was considered to be pH 5.25 (phosphate buffer).

6.2.3 Optimal Temperature

The analysis of the temperature dependence upon the enzymatic reaction gives information considering the heat inactivation of the enzyme. At elevated temperatures, the irreversible denaturation of the protein occurs with a direct influence over the biocatalytic process. The maximum activity for a biocatalyst is reached for a so-called optimum temperature, after this point the reduced activity indicates the protein denaturation. It is in fact only an apparent value, since the

reaction time chosen for enzymatic treatment also determines the optimum temperature at which the yield of product is maximum. The apparent temperature optimum for a biocatalyst increases with the decrease of reaction time in the test. Therefore, for an enzyme-catalyzed reaction it is advisable to proceed at lower temperature than the optimal one *(Wiseman, 1975)*.

To determine the temperature optimum of recombinant Inulase II, similar experiments as for optimum pH were performed. For a pH of 5.25 (phosphate buffer), the enzymatic reaction was performed at different temperatures in a range from 30 to 85°C. Except the reaction temperature, the activity test follows the standard procedure described in Chapter 8, section 10 (reaction time 30 minutes). The DFAIII concentrations, which can be directly correlated with the enzyme activities, were determined by HPLC. Their correlation the reaction temperature is presented in figure 6.4.

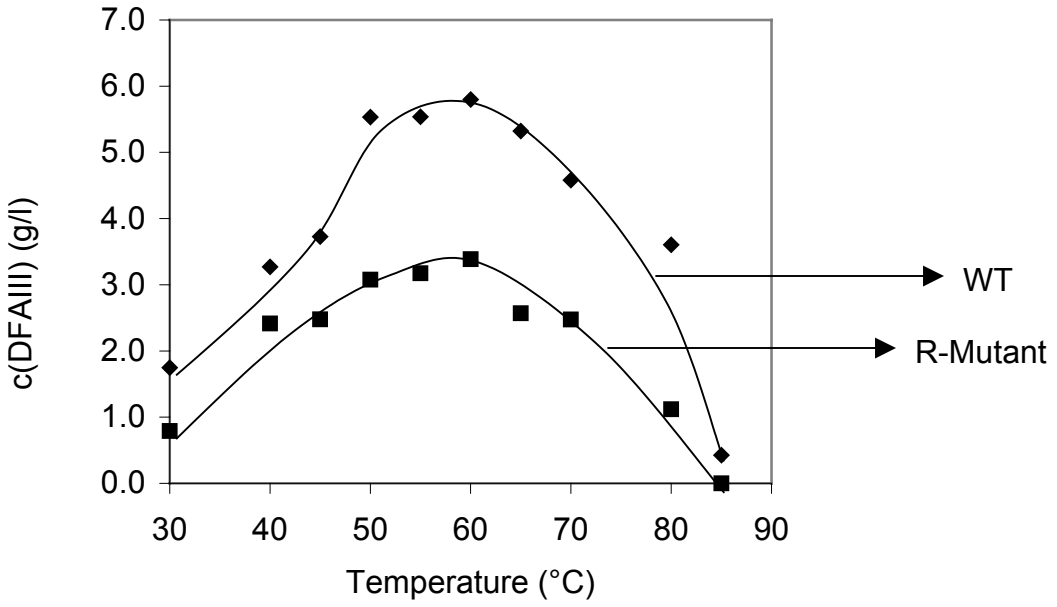


Figure 6.4. Effect of temperature on the DFAIII formation using *E.coli* Inulase II enzyme solutions. (pH 5.25, Dahlia inulin concentration 100 g/L).

In figure 6.4. the influence of the reaction temperature upon the DFAIII formation is presented for both enzyme solutions: *E.coli/*pMS*ift*OptWT and *E.coli/*pMS*ift*OptR. As it can be seen, the temperature optimum is about 60°C in both cases. After 70°C a rapid decrease of the DFAIII formation was observed, probably due to enzyme denaturation. For further utilization of recombinant Inulase II no higher temperatures than 60°C were used.

6.2.4 Temperature Stability

The stability of an enzyme is one of the most important properties of the biocatalytic system. It is difficult to determine the behaviour of an enzyme over extended periods of time under process conditions *(Boy, 2001)*. To investigate the *E.coli* Inulase II thermo-stability, the enzyme solutions were incubated at 30°C, 60°C and 65°C. The enzymatic assay was carried out using 100 µl aliquots of the treated enzyme solution and performing the standard activity test. The results are presented in figure 6.5, A for *E.coli/*pMS*ift*OptWT enzyme solution and B for the *E.coli/*pMS*ift*OptR, respectively.

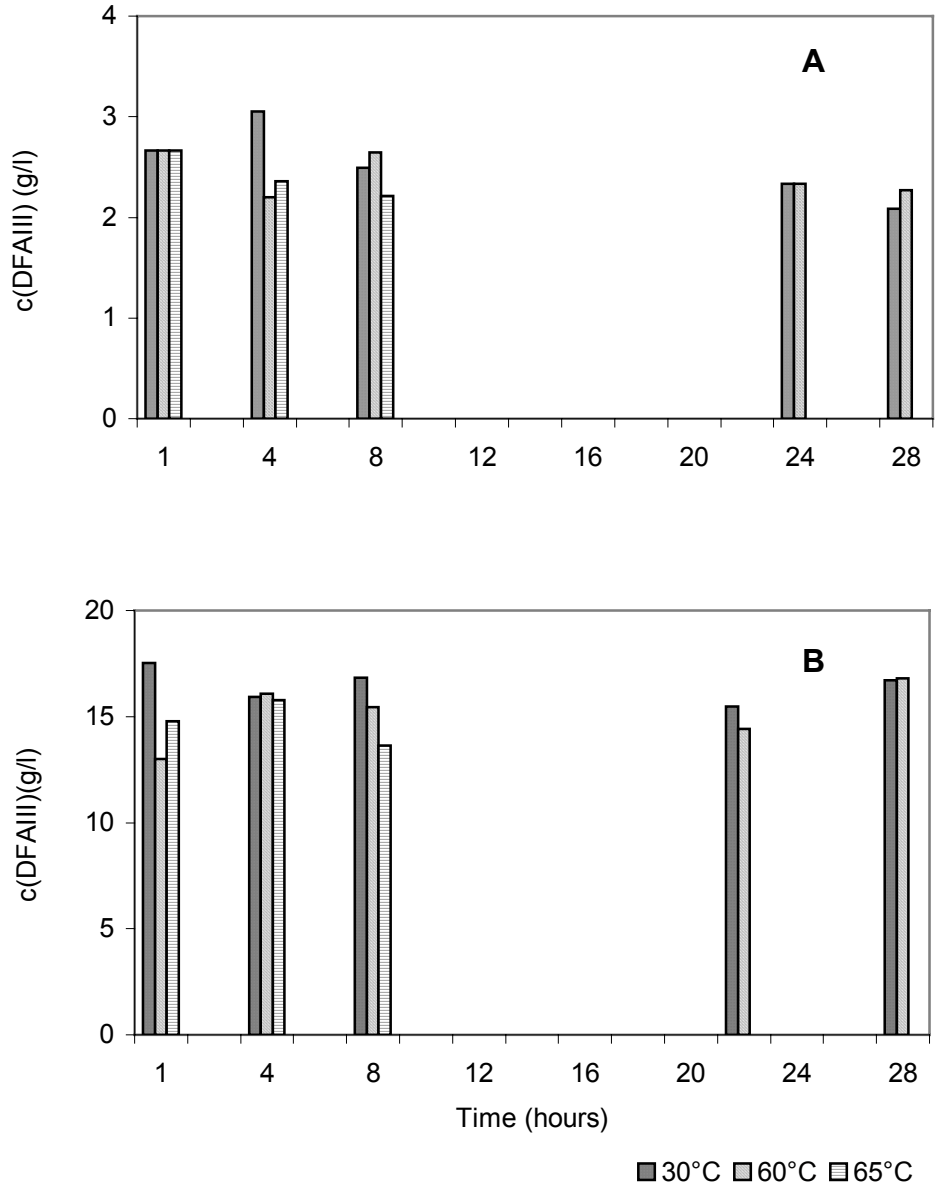


Figure 6.5. Temperature stability of *E.coli* Inulase II. (A): *E.coli/*pMS*ift*OptWT enzyme and (B): *E.coli/*pMS*ift*OptR enzyme (0.04 M phosphate buffer pH 5.25, T=50°C, Cosucra inulin concentration 100 g/L)

As it can be seen in the figure 6.5 (A and B), the stability of both enzyme solutions were followed for 28 hours, recording the DFAIII concentrations obtained after activity test. During the experiment, no significant variations in the DFAIII concentration could be detected when the solutions incubated at 30°C

and 60°C were investigated. Not expecting the enzyme to be stable at higher temperatures over extended periods of time, the experiment for the enzyme solutions temperated at 65°C was stopped after 8 hours. Considering the solutions incubated at 30°C as control solutions, no enzyme deactivation due to increased temperature can be stated. Both enzyme solutions (*E. coli/*pMS*ift*OptWT and *E. coli/*pMS*ift*OptR) are stable up to 28 hours at 60°C.

6.2.5 Michaelis-Menten Kinetics

In order to determine the Michaelis-Menten parameters for the *E.coli* Inulase II, experiments were performed varying the inulin concentration in the inulin solution (see. 9.10) in the range of 30 to 120 g/L. The activity test was carried out at 50°C and pH 5.25 (phosphate buffer). The DFAIII concentrations were recorded after 5, 10, 20 and 30 minutes reaction time and were used to calculate the reaction rate.

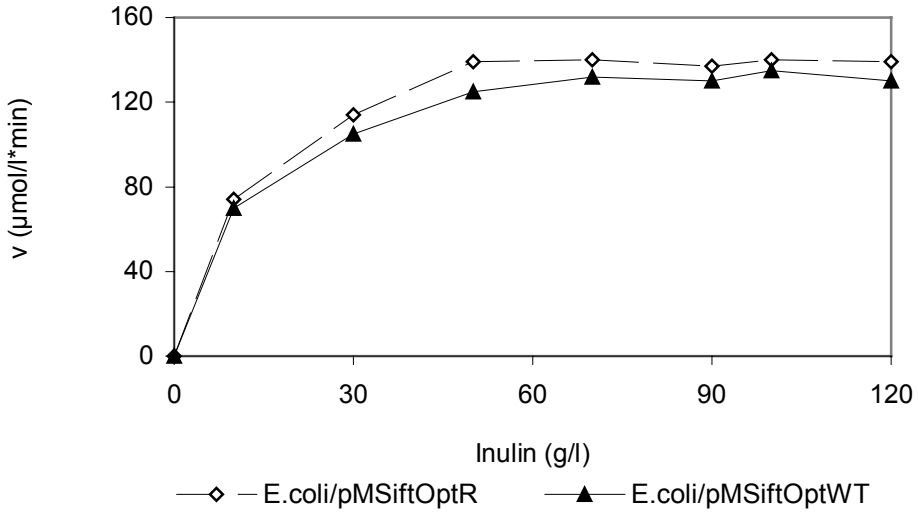


Figure 6.6. Substrate saturation curve. (T=50°C, 0.04 M phosphate buffer, pH 5.25, Cosucra inulin)

The profile of the curves is similar for both enzyme solutions investigated and substrate saturation was reached at around 70 g/L inulin concentration. In order to achieve the maximum reaction rate, an enzymatic reaction should be performed with substrate concentrations in the range of saturation. The standard activity test employed fulfills this condition with 100 g/L inulin concentration.

In order to determine the Michaelis-Menten parameters (K_M and maximum reaction rate (v_{max})), the following considerations had to be assumed:

- the inulin is a mixture of oligomers (DP – medium degree of polymerization - 9.4) and the depolymerizing reaction rate is the same for all oligomers *(Uchiyama, 1975)*,
- the inulin molecular weight was considered to be 1,539 g/mol,
- the Cosucra inulin contains only 84.2% pure inulin *(Neubauer, 1998)*.

The kinetic parameters were determined using the Michaelis-Menten equation but the hyperbolic profile of the curves affect the accuracy of the data and so the Hanes-Woolf linearization (linear regression) (figure 6.7) was additionally used.

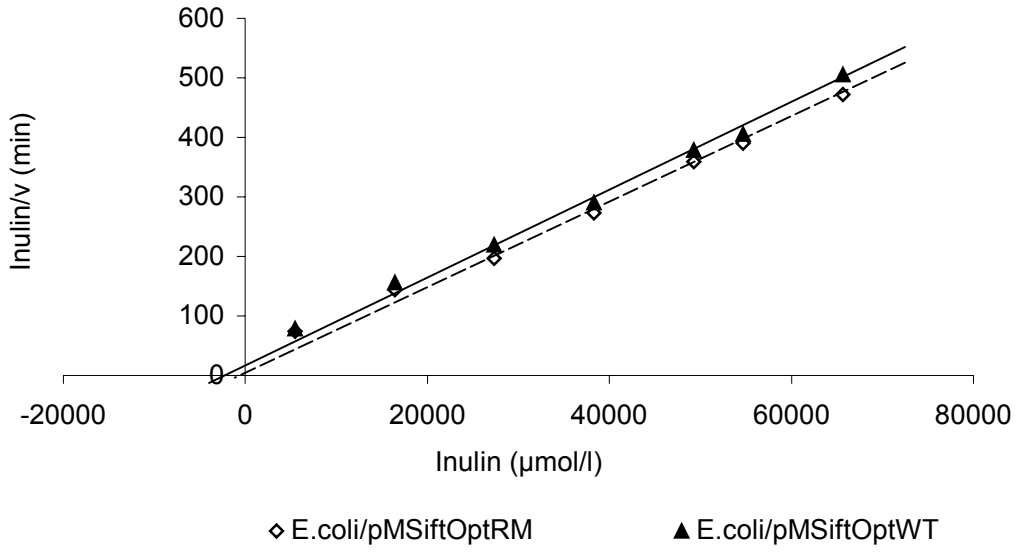


Figure 6.7. Hanes-Woolf linearization for kinetic parameter determination.
(the linearization was made for the same experimental data from figure 6.6)

The results obtained are summarized in table 6.1.

Table 6.1. Michaelis-Menten parameters (K_M and v_{max}) for *E. coli/*pMS*ift*OptWT and *E. coli/*pMS*ift*OptR enzyme solutions. (K_M in mmol/L and v_{max} in µmol/(L×min))

Enzyme solution	Michaelis-Menten		Hanes-Woolf		Runge-Kutta	
	K_M	v_{max}	K_M	v_{max}	K_M	v_{max}
*E.coli/*pMS*ift*OptWT	5.2	130	5.1	144	5.4	126
*E.coli/*pMS*ift*OptR	4.8	140	4.5	151	4.9	1 18

There is no significant disagreement between the results obtained using both the Michaelis-Menten equation and the Hanes-Woolf liniarization. Small differences can be observed between the two enzyme solutions under investigation. For *Arthrobacter* sp. Bu0141 Inulase II a K_M values of 2.3 mmol/L (for inulin molecular weight 5,120 g/mol) *(Jahnz, 2001)*, and of 5.4 mmol/L (the inulin molecular weight 1,539 g/mol) were found *(Walter, 2000)*.

Along with the methods presented above, the enzymatic parameters were supplementary determined using the Runge-Kutta integration procedure. This method is supported by a computer program developed by Demuth *(Demuth, 1994)*. The calculation errors are eliminated and so their influence over the final result is minimized. The K_M values obtained using this method agree with the one obtained by classical methods.

6.3 Characteristics of Immobilized Inulase II

The changes in the stability and kinetic parameters of an enzyme can be stated only carrying out experiments with the immobilized biocatalyst in the same conditions as for the free enzyme *(Buchholz, 1987)*. In order to investigate the temperature stability and to determine the kinetic parameters, standard activity tests for immobilized enzyme were performed (see 8.10.1.3). The Inulase II enzyme solutions employed for immobilization were obtained after performing a 20 L mass-culture medium-fermentation experiment and cell disruption using the high-pressure homogenizer (9.6.3). The immobilization was carried out according to the standard procedure described in chapter 9.9. As carrier, Duolite A 568 was employed and, to reach the maximum loading of the resin, *E. coli/*pMS*ift*OptWT and *E. coli/*pMS*ift*OptR enzyme solutions having an activity of 60,000 $U/L_{solution}$ were assayed for immobilization. The immobilized activities obtained were 100,000 U/L_{resin} for *E. coli/*pMS*ift*OptWT Inulase II and 130,000 U/L_{resin} for *E. coli/*pMS*ift*OptR enzyme.

6.3.1 Temperature Stability

In addition to the other net advantages of immobilizing enzymes (see 5.1) (as the recovery of the biocatalyst from the reaction mixture and the possibility to perform continuous bioprocesses) the thermostability of the enzyme can be improved.

To obtain comparable results with the data obtained for soluble enzyme, the thermostability of immobilized *E.coli* Inulase II enzyme solutions was also investigated incubating the immobilized biocatayst at 30°C, 60°C and 65°C. The immobilized activity was 130,000 U/L_{resin} for *E. coli/*pMS*ift*OptR and 100,000 U/L_{resin} for *E. coli/*pMS*ift*OptWT enzyme solution. 2 g incubated immobilized enzyme were supplied with 20 ml inulin solution, pH 5.25 at 50°C for activity test

and the enzymatic reaction was stopped after 10 minutes. The DFAIII concentrations were followed for 480 hours and the results obtained are presented in figure 6.8 (A) for *E. coli/*pMS*ift*OptWT immobilized enzyme and (B) for *E. coli/*pMS*ift*OptR immobilized enzyme.

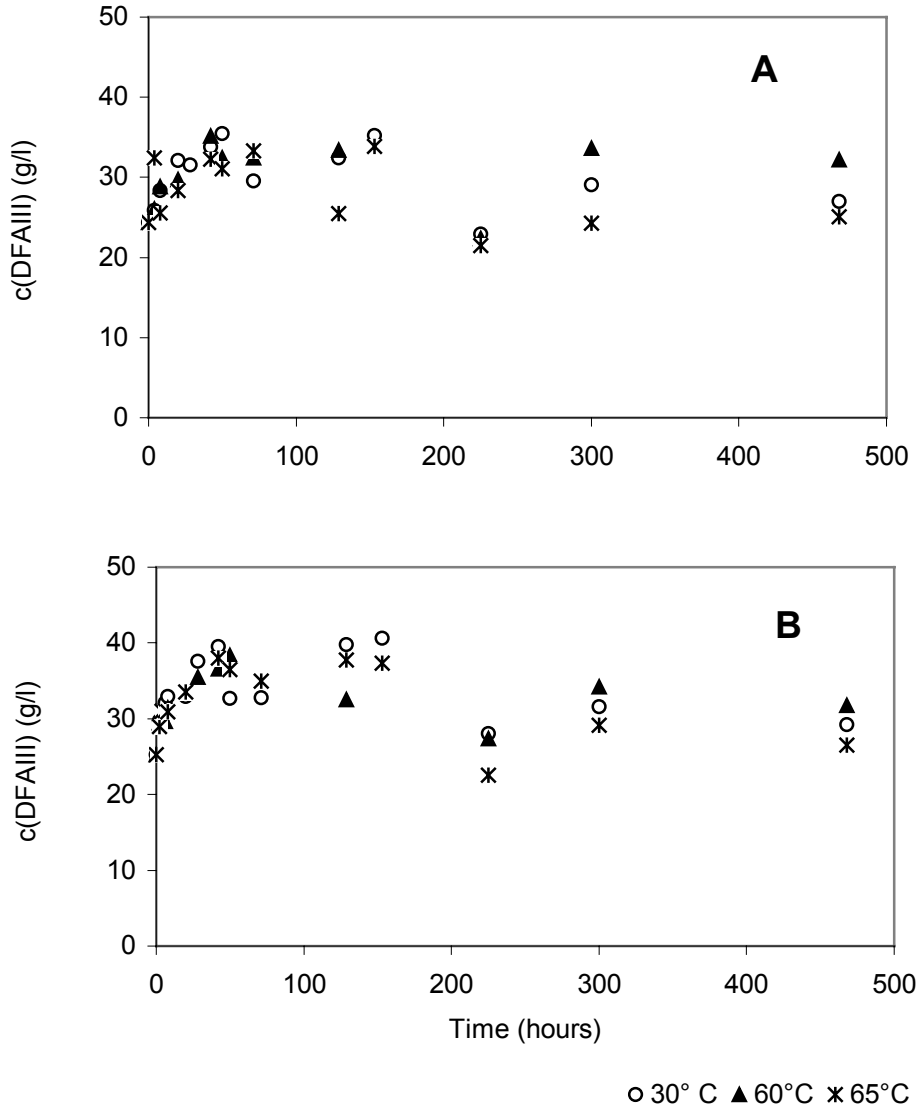


Figure 6.8. Temperature stability of immobilized *E.coli* Inulase II. (A): *E.coli/*pMS*ift*OptWT enzyme and (B): *E.coli/*pMS*ift*OptR enzyme (Duolite A 568 resin equilibrated with phosphate buffer pH 9, activity test parameters: 0.04M phosphate buffer pH 5.25, T=50°C, Cosucra inulin concentration 100 g/L)

Using as reference the stability of the immobilized enzyme incubated at 30°C, no enzyme deactivation could be observed in time at 60°C or 65°C. The DFAIII concentrations recorded in the first 50 hours are surprisingly increasing without an obvious explanation. Further, a constant profile of the curves is maintained en route of the experiment for all three temperatures investigated. A clear comparison between the soluble and immobilized enzymes is not possible since the stability of the soluble enzymes were investigated for only 28 hours and so a statement regarding the improvement of the temperature stability due to immobilization cannot be made. Both immobilized enzymes (*E. coli/*pMS*ift*OptWT and *E. coli/*pMS*ift*OptR) are reacting similarly with regard to the heat treatment and this prolonged stability makes them suitable for a process in the technical scale carried out at temperatures as high as 65°C.

6.3.2 Michaelis-Menten Kinetics for Immobilized Inulase II

Due to enzyme immobilization, variations in the kinetic parameters values could be possible since the microenviroment in the immediate vicinity of the enzyme differs for immobilized to soluble biocatalyst *(Lee and Woodward, 1983)*. A decrease in K_M value of the enzyme (the resulting value is referred to as apparent K_M) can have practical advantages since the rate of reaction will be increased at lower substrate concentrations. On the other hand, an increase in the K_M value upon immobilization means that a higher substrate concentration is required to achieve the same rate of reaction as for the soluble enzyme.

To determine the Michaelis-Menten parameters of immobilized *E. coli/*pMS*ift*OptWT and *E. coli/*pMS*ift*OptR enzymes, the immobilization was performed following the standard procedure (see 8.9) using 60,000 $U/L_{solution}$ (to insure the maximum loading of the resin). The immobilized activity obtained was 100,000 U/L_{resin} for *E.coli/*pMS*ift*OptWT enzyme and 130,000 U/L_{resin} for *E.coli/*pMS*ift*OptRM enzyme. The investigations were carried out using 3, 6, 50

and 100 g/L inulin concentrations. These values were chosen accordingly to the K_M values determined for soluble enzymes. The enzymatic reaction was carried out with 2 g immobilized enzyme and 20 ml inulin solution (0.04 M phosphate buffer, pH 5.25) at 50°C. Samples were collected after 5, 10, 15, 20, 30, 45 and 60 minutes and the DFAIII concentrations were determined using HPLC analysis.

The kinetic parameters calculated applying the Hanes-Woolf linear regression agree with the one obtained by Runge-Kutta integration procedures. In table 6.4, the results obtained are compared with the values for soluble enzymes.

Table 6.2. Comparison of kinetic parameters for soluble and immobilized *E.coli* Inulase II (Hanes-Woolf lineary regression)

Enzyme solution	**Soluble enzyme**		**Immobilized enzyme**	
	K_M (mmol/L)	**v_{max}** (µmol/(L×min))	**K_M** (mmol/L)	**v_{max}** (µmol/(L×min))
*E.coli/*pMS*ift*OptWT	5.1	144	5.6	200
*E.coli/*pMS*ift*OptR	4.5	151	4.9	167

The K_M values are slightly increased upon immobilization and also the reaction rate. Consequently higher substrate concentrations are required for the enzyme saturation. However, the differences between the soluble and immobilized form are not significant because they are in the error range caused by the cell disruption procedure and the activity test (see 3.3.2).

6.4. Discussion

Recombinant Inulase II was examined for size and different kinetic parameters. The molecular weight of *E. coli/*pMS*ift*OptWT enzyme was estimated to 46 kDa by performing SDS-Page gel-electrophoresis and the result fits with the value obtained by sequencing the DNA of native Inulase II (45.2 kDa). Since the only difference at primary structure level consists in the replacement of one amino-acid residue (arginine instead of glycine), the *E. coli/*pMS*ift*OptR enzyme is assumed to have the same molecular weight as *E. coli/*pMS*ift*OptWT enzyme. Similar results were reported for Inulase II from other sources (see table 6.3). For Inulase II isolated from *Arthrobacter globiformis* C11-1 a molecular weight of 45 kDa was determined by SDS-Page electrophoresis *(Haraguchi et al., 1988)*. Investigating the enzyme from *Arthrobacter* sp. H65-7, Yokota found a molecular weight of 49 kDa *(Yokota, 1991, 1)* by SDS-polyacrylamide gel electrophoresis and 100 kDa by gel filtration. These results suggested that the enzyme is a dimer. It had been reported that also the Inulase II from *Arthrobacter ilicis* OKU17B is a dimer. *(Kawamura et al., 1988).*

The effect of pH, temperature, and substrate concentration was investigated for both soluble and immobilized inulase and the results were compared with the data reported in literature.

Considering the results reported in literature for the optimum pH for the enzymatic reaction, a pH range between 4 and 7.5 was investigated for the optimal pH value for *E.coli* Inulase II. This range was covered using two buffer solutions, 0.1 M acetate buffer for pH values $<$ 5 and 0.04 M phosphate buffer for pH values $>$ 5. A 5.25 pH (phosphate buffer) was found as the optimum value for the enzymatic reaction. As it can be seen in table 6.3, this is a result comparable with the ones stated in literature.

The DFAIII concentrations obtained for different buffers having the same pH (see figures 6.2. and 6.3.) suggested that the ion-nature is influencing the enzyme activity. The effect of various metal ions as Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+},

Hg^{2+}, Fe^{2+}, Ni^{2+} over the activity of Inulase II isolated from *Arthrobacter* sp. H65-7 was investigated but no significant influence could be stated *(Yokota et al., 1991, 1)*.

The optimum temperature of *E.coli* Inulase II was determined performing the standard activity test varying only the temperature of the enzymatic reaction between 30 and 85°C. The optimal temperature was found to be around 60°C, a rather high value considering that, for the majority of enzymes, the optimal temperature value lies between 40 and 50°C *(Bisswagner, 1979; Lüthje, 1990)* but, on the other hand, is comparable with the optimum temperature reported for Inulase II isolated from other strains (see table 6.3). A rapid deactivation was noticed for temperatures higher than 70°C and so the enzyme was not subjected to temperatures higher than 60°C for the enzymatic reaction.

The temperature stability is one of the most important characteristics of a biocatalyst. Different approaches were reported in 1989 by Sadana who proposed an empirical stability index for enzyme deactivation *(Sadana, 1989)* and by Boy, who developed a method for the accelerated measurement of the activity and stability of enzymes *(Boy, 1999)*. To obtain information in a short period of time to characterize the biocatalyst over extended range of temperature, the enzyme ageing process was accelerated by increasing continuously the temperature during the experiment. The evaluation procedure involves the assuming of a deactivation mechanism, formulating the corresponding mathematical model, determining the model parameters and so calculating the biocatalyst characteristics *(Boy, 1999)*. In 1997, a method that correlates the enzyme thermo-inactivation with the enzyme mechanism and the kinetic model, containing the temperature dependence of reaction rate constants according to Arrhenius equation, was stated *(Vrabel, 1997)*.

The behaviour of soluble and immobilized *E.coli* Inulase II was tested at three different temperatures (30°C, 60°C, 65°C). As a consequence of immobilization an increase in the heat stability was expected. For instance, a 3~4 fold increase in the heat stability was stated for glucose amylase immobilized on aminated

silica gel *(Nishimura et al., 1990)*. An important increase in the thermal stability was also reported for endoinulase from *Streptomyces* sp. S56 *(Kim and Chang, 1992)*, as a result of DEAE-cellulose immobilization. Consequently, the immobilized Inulase II was investigated over a longer period of time (480 hours) compared with the soluble enzyme (28 hours). Considering the 30°C as reference, no enzyme deactivation due to temperature increase was detected even at 65°C, so the enzyme is a thermo-stable one. Thermo-stability studies were carried out for Inulase II isolated from other strains. The values reported are varying between 50°C for *Arthrobacter ureafaciens* Inulase II *(Uchiyama et al., 1973)* and 75 °C for the enzyme isolated from *Arthrobacter* sp. H65-7 *(Yokota et al., 1991, 2)* (see table 6.3) This temperature stability up to 75°C makes the enzyme suitable for industrial production of DFAIII and was determined after keeping the enzyme solution at various temperatures for 20 minutes.

The Michaelis-Menten parameters were investigated for both soluble and immobilized enzyme solutions. Different approaches were adopted in order to calculate the Michaelis-Menten constant and the reaction rate: non-linear regression, Hanes-Woolf linearization and Runge-Kutta integration method. The results obtained are summarized in table 6.1 (for soluble enzyme solutions) and 6.2 (for immobilized enzyme solutions). The K_M value and the reaction rate for *E. coli/*pMS*ift*OptR enzyme are slightly higher than the respective parameters for *E. coli/*pMS*ift*OptWT enzyme solution, suggesting higher substrate specificity. These differences can be attributed to the environmental effects in the vicinity of the immobilized enzyme particles, as for instance the adhering solvent layer in the surrounding of the particles (Nernst layer), in which the concentration of the substrate is lower than in the total solution. Furthermore intra-particle diffusion may cause decreasing profiles of substrate concentration inside the particles. Consequently, a higher substrate concentration will be required to saturate the enzyme with substrate *(Woodward, 1984)*.
Summarizing, both *E.coli/*pMS*ift*OptWT and *E.coli/*pMS*ift*OptR enzyme solutions are acting similarly at the operational conditions investigated.

Table 6.3. Characteristics of Inulase II isolated from different strains.

Strain (references)	kDa[1)]	kDa[2)]	pH[3)]	T(°C)[4)]	K_M/M_W[5)]
1. *A. globiformis* C11-1 (Haraguchi et al., 1988)	45	50	5.0	55	
2. *A. ureafaciens* (Uchiyama et al., 1973)	6-7	50			
3. *A. ilicis* OKU17B (Kawamura et al., 1988)	27	50	5.5	60	
4. *Arthrobacter* sp. H65-7 (Tomita et al., 1991)	49	100	5.5	60	0.8/5,000
5. *A. ureafaciens* ATCC 21124 (Neubauer, 1998)			5.25	70	3.0/1,539
6. *Arthrobacter* Bu0141 (Jahnz, 2000)	45		5.0		2.3/5,120
(Walter 2000)					5.4/1,539
7. *E. coli*/pMS*ift*OptWT (this work)	46		5.25	60	5.1/1,539
8. *E. coli*/pMS*ift*OptR (this work)	46		5.25	60	4.5/1,539

[1)] Molecular weight determined by SDS-Page electrophoresis

[2)] Molecular weight determined by gel filtration

[3)] Optimum pH for enzyme-catalyzed reaction

[4)] Optimal temperature for enzyme-catalyzed reaction

[5)] K_M value, in mmol/L and molecular weight of inulin, in g/mol

Chapter 7

Summary and Conclusions

The recombinant *E. coli* strains *E. coli*/pMS*ift*OptWT (DSMZ 13463) and *E. coli*/pMS*ift*OptR (DSMZ 13465) were investigated in the frame of this work.

The recombinant inulase II is accumulated intracellularly and in order to isolate it it, the *E. coli* cells have to be disrupted. Different methods were used for this purpose: grinding with glass beads as well as ultrasonication for small-scale disruption and a high-pressure homogenizer to obtain larger volumes of stock enzyme solutions. The disruption parameters were established and the results obtained for all three methods are reliable and comparable.

In order to obtain high volumetric activities for recombinant *E. coli* enzyme solutions, fermentation experiments up to 20 l bioreactors scale were performed employing batch or fed-batch strategies investigating different media containing yeast-extract, glycerol, mineral elements and vitamins, with the aim to insure a proper culture development. The feeding solutions also contained yeast extract and glycerol and the different fed-batch strategies adopted were selected in order to maintain C-limiting conditions and low acetate concentrations, witch inhibit the culture development and the recombinant protein formation.

The strains *E. coli/*pMS*ift*OptR and *E. coli/*pMS*ift*OptWT were cultivated in small scale (5 ml) LB-Medium. This led to OD values around 4 and enzyme activities of about 350,000 U/L.
LB Medium was also employed for cultivations in 1 L fermenters. However improved aeration and pH control did not lead to an increase of activity, and enzyme activities of about 350,000 U/L were reached as well.

To develop high-cell density cultivation with a cheap medium feasible for technical scale, different media were tested. *E. coli/*pMS*ift*OptR strain was assayed for fermentation experiments using a yeast-extract/glycerol medium. A cultivation in a 20 L bioreactor was carried out with a continuous fed-batch profile wich followed the glycerol concentration in the cultivation medium. Although excess acetate formation was avoided by different feeding strategies, no higher values than 750, 000 U/L could be obtained. At the end of experiment values around 25 and 8,5 g/L were detected for optical density and dry cell weight, respectively. Therefore other media were investigated.
A different feeding approach was adopted for mineral / yeast-extract medium fermentation. The fed-batch strategy was aimed to maintain C-limiting conditions and had the dissolved oxygen content of the medium as control parameter. The culture developed exponentially both in the batch and the fed-batch phases but with a lower growth rate. However an early cellular lysis occurred after 20 hours of fermentation, which leads to an increased base and antifoam consumption. Therefore, the exceptionally high values obtained for optical density and dry cell weight (100 OD_{600} and 50 g/L respectively) are questionable. High enzyme activities were obtained, around 3,000,000 U/L but a clear trend of the activity variation could not be stated. To the end of fermentation course a significant loss of activity occurred (~ 1,000, 000 U/L). Due to tight time schedule this mineral / yeast-extract medium was not further investigated, since another suitable medium for high-cell density fermentation should be evaluated for cultivation of *E. coli/*pMS*ift*OptR.

Further fermentation experiments were performed using the so-called mass-culture medium which contains yeast-extract and glycerol as carbon and nitrogen sources along with salts and trace elements *(Riesenberg et al., 1991)*. The control of the feeding profile took into consideration both the dissolved oxygen concentration and the glycerol depletion from the culture medium. Experiments were performed in 1 L bioreactor employing a punctual feeding and in a 20 L bioreactor when continuous and exponential feeding profiles were investigated. For the experiment performed at 1 l bioreactor scale, a peak was observed for the enzyme activity (2,000,000 U/L) immediately after the feeding point but at the end of the experiment only values around 1,000,000 U/L were recorded. The loss of activity detected for the fed-batch cultures was considered to be due to cell lysis, but no significant free enzyme amounts were detected in the culture broth: the values around 80,000 U/L do not compensate for the lack of 1,000,000 U/L inulase activity. However the enzyme could be present as inactive aggregates (inclusion bodies) in the cell debris. One possible reason for this loss of activity could be the formation of acetate and other metabolic side products, which inhibit further growth and enzyme production. The small reactor volume employed here made it impossible to carry out a slow and nutrient-limiting feeding, therefore a continuous feeding strategy was examined in a larger scale (20 L) fermenter.

Scaling-up, continuous and exponential feeding strategies were investigated for the mass-culture medium in a 20 l bioreactor. Both methods intended to minimize the formation of acetate as a by-product. The feeding solutions contained glycerol and yeast-extract.

For the continuous feeding experiment, the fed-batch procedure was set to follow the glycerol depletion from the culture medium and to maintain a value around 20% for oxygen saturation in the culture broth during the fermentation experiment. The highest enzyme activity (~ 1,100,000 U/L) was recorded after 12 hours of fermentation around the initiation of the fed-batch procedure. After this moment, even though the OD increased further a loss of activity was observed. To the end of the fermentation, an overall decrease of 70% was detected,

meaning that only an activity of 310,000 U/L could be recovered from the fermentation broth. Again, no high amounts of enzyme could be identified in the culture broth as free enzyme due to cell lyses.

A totally different strategy was employed trying to control the culture development and to avoid the loss of enzyme activity till the end of experiment: exponential feeding. A reduced growth rate can lead to the appropriate conditions for increased recombinant protein production. Moreover, acetate accumulation in the culture medium can be prevented using limiting growth conditions. The feeding solution was pumped into the fermenter at an appropriate flow rate to obtain a reduced growth rate for *E. coli/*pMS*ift*OptR (μ=0,12) compared with the one calculated from previous experiments under unlimited conditions (μ=0,25). In addition, a 20% dissolved oxygen saturation was maintained in the cultivation medium during the course of fermentation. The fed-batch procedure was initiated as a response to a drop in dissolved oxygen concentration below 20%. After a high value recorded immediately after the fed-batch procedure started (~ 2,000,000 U/L), a rapid loss of inulase activity occurred, a decrease of 50 % being recorded in less than 8 hours. For the last 15 hours of cultivation the downwards tendency is maintained but at lower rates, at the end of experiment only 550,000 U/L volume activity could be obtained. Investigating the presence of recombinant inulase II as free enzyme in the culture medium as a result of cell lyses, 80,000 U/l could be detected. This value covers only 5% of the lacking enzyme activity.

Even with exponential feeding, the loss of activity could not be circumvented and also no high cell densities were obtained. Further experiments are needed to determine which factor triggers this switch in metabolism, leading to a reduced growth rate and a significant loss of recombinant enzyme.
One possible explanation why inulase activity is decreasing so fast is the action of *E. coli* proteases becoming active due to cell lysis. This lysis may be caused by growth conditions (over-supply or lack of nutrients). As mentioned above the

reasons for this switch are not clear at the moment, however, the proteolytic cleavage can be inhibited by heat treatment. To prevent the actions of intracellular E.coli proteases, the bioreactors were heated at 60°C for 30 minutes. This temperature increase is supposed to destroy the proteases but has no influence on the recombinant inulase II, which is stable up to 65°C.

This procedure was carried out for fermentation of both *E. coli/*pMS*ift*OptR and *E. coli/*pMS*ift*OptWT strains, in order to obtain high activity stock recombinant enzyme solutions. As a result enzyme solutions with an activity of 1,500,000 U/L for *E. coli/*pMS*ift*OptR and 240,000U/L for *E. coli/*pMS*ift*OptWT were obtained. This is considered as a good result for technical application of the enzyme.
The loss of activity while cultivating recombinant *E. coli* strain could be observed in later fermentation stages (~ 12 hours), it does not seem to be a consequence of the medium or the fed-batch strategy, since it occurred under differing conditions. External proteases as a source of enzyme loss could be excluded by changing the experimental procedure. Another reason could be the formation of so-called inclusion bodies (inactive aggregates), a problem which is often encountered with recombinant proteins which accumulate in high amounts. Further investigations are necessary to elucidate the loss of activity occurring in late stages of fermentation experiments.

For the immobilization of recombinant E. coli inulase II weakly basic anion-exchangers resins were investigated. The best results were obtained for Duolite A 568 and Amberlite 94 S where the yield of the immobilization is around 20% and around 19,5%, respectively.

Adsorption experiments were performed with Duolite A 568 in order to establish the maximum loading of the resin. Around 50% of the initial activity was found as immobilized activity for very low enzyme loading. A rapid decrease in the activity yield was observed with increasing enzyme loading, while the immobilized activity remained constant at around 110 U/ml_{resin}. Investigating the free enzyme,

low enzyme activities were detected for the unbound enzyme, which do not fit into the activity balance. It was possible to recover 100% of enzyme activity. One reason may be that not all immobilized enzyme is able to participate in the enzymatic reaction due to steric hindrance.
In order to avoid the desorption under conditions to which the enzyme is subjected under reaction conditions, a subsequent immobilization step (cross-linking with glutardialdehyde) was performed. For the immobilization of the *E.coli* Inulase II on anion-exchangers resins, the cross-linking with a 3 g/L glutardialdehyde solution was efficient and did not lead to enzyme deactivation.

The half-life of immobilized *E.coli/*pMS*ift*OptR enzyme solution was established investigating the behaviour of the immobilized biocatalyst for the repeated biotransformation of inulin. A 50% decrease in the biocatalytic activity of immobilized *E.coli/*pMS*ift*OptR enzyme solution occurred after 11 days.

Recombinant Inulase II was examined for size and different kinetic parameters. The molecular weight of *E. coli/*pMS*ift*OptWT enzyme was estimated to 46 kDa by performing SDS-Page gel-electrophoresis and the result fits with the value obtained by sequencing the DNA of native Inulase II (45.2 kDa). Since the only difference at primary structure level consists in the replacement of one amino-acid residue (arginine instead of glycine), the *E. coli/*pMS*ift*OptR enzyme is assumed to have the same molecular weight as *E. coli/*pMS*ift*OptWT enzyme.

The effect of pH, temperature, and substrate concentration was investigated for both soluble and immobilized inulase. The optimal values for enzymatic reaction were determined to be 5.25 (phosphate buffer) and 60°C for pH and temperature, respectively.

The temperature stability of soluble and immobilized *E.coli* Inulase II was tested at three different temperatures (30°C, 60°C, 65°C). Considering the 30°C as

reference, no enzyme deactivation due to temperature increase was detected even at 65°C, so the enzyme is a thermo-stable one.

The Michaelis-Menten parameters were investigated for both soluble and immobilized enzyme solutions. Different approaches were adopted in order to calculate the Michaelis-Menten constant and the reaction rate: non-linear regression, Hanes-Woolf linearization and Runge-Kutta integration method. The results obtained are summarized in table 6.1 (for soluble enzyme solutions) and 6.2 (for immobilized enzyme solutions). The K_M value and the reaction rate for *E. coli/*pMS*ift*OptR enzyme (4.8 mmol/L and 151 µmol/L*min) are only slightly different than the respective parameters for *E. coli/*pMS*ift*OptWT enzyme solution (5.2 mmol/L and 144 µmol/L*min).
Summarizing, both *E.coli/*pMS*ift*OptWT and *E.coli/*pMS*ift*OptR enzyme solutions are acting similarly in the operational conditions investigated.

Chapter 8

Methods and Materials

1. Microorganisms

All cultivation experiments were performed with genetically modified *Escherichia coli* strains carrying the gene for inulinase II from *Arthrobacter spec*. BuO 141. These strains were obtained from the group of Prof. Vorlop (FAL) as a result of a FNR joined project between the FAL and Zuckerinstitut.

Table 9.1. *Escherichia coli* strains

Strain	DSMZ	Plasmid
Escherichia coli XL-1 blue	13463	pMS*ift*OptWT
Escherichia coli XL-1 blue	13465	pMS*ift*OptR

Besides the microorganisms mentioned above, an *Escherichia coli* XL1-blue (Fa. Stratagene Corp., La Jolla, California, USA) strain was used, but only for cell disruption investigations.
The genotype of *Escherichia coli* XL-1 blue is: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F`*proAB lacI* ^{q}*Z ⊿M15* Tn*10 (*Tet^{r}*)*].

2. Media for Cultivation

2.1. Strain Storage

2.1.1. Long Time Storage (-80°C)

For long time storage of genetically modified *E. coli* (–80°C), bacterial cells are suspended in equal volumes of LB medium + ampicillin (see 8.2.2.1) and sterile glycerol (87%) (8.3.3.1).

2.1.2. Short Time Storage (on Petri Plates)

To have fresh single colonies for immediate use, the culture was streaked every three weeks on perti plates with LB–Agar medium.

LB medium + ampicillin	
Agar	15 g/L

2.2. Cultivation

The optimum cultivation parameters are: 37°C and pH 7. All experiments with the strains carrying ampicillin resistance were performed in the presence of 60 mg/ml ampicillin solution (ratio 1:1000 between ampicillin and culture medium).

2.2.1. LB (Luria Bertani) Medium *(Sambrook et al., 1989)*

Yeast-extract	5 g/L
Peptone from Casein (pancreatic digest)	10 g/L
NaCl	10 g/L
pH adjusted to 7 with NaOH 1 M	

The medium was sterilized at 121°C for 20 minutes (Varioklav, Fa. H+P Labortechnik GmbH, Oberschleissheim). After sterilization, the vessel containing the medium was temperated at 50°C for one hour using a water-bath. Sterile ampicillin solution (with a concentration of 60 mg/ml) was added for strain selection (*E.coli* XL-1 blue/pMS*ift*OptWT and *E.coli* XL-1 blue/pMS*ift*OptR are carrying an ampicillin resistance gene) in a ratio of 1:1000.

Aqueous ampicillin solution is sterile filtered using 0,22 µm filters (Millex-GS) and stored at –20°C.

2.2.2. Modified LB (Luria Bertani) Medium

Yeast-extract	10 g/L
Peptone from Casein (pancreatic digest)	20 g/L
NaCl	5 g/L
pH adjusted to 7 with NaOH 1 M	

This medium was used to obtain the second preculture for mineral-yeast-extract fermentation experiments. The medium preparation and sterilization follows the description from unmodified LB medium.

2.2.3. Yeast extract-glycerol Medium *(Jahnz, 2001)*

The fed-batch fermentation experiment started with a medium containing yeast-extract and glycerol. The feeding profile was conducted according to the glycerol depletion.

A. Medium

Yeast-extract (technical)	15 g/L
Glycerol	2.5 g/L

B. Feeding solutions

	Concentration in 20 L bioreactor
B.1 Yeast-extract	50 g to 10 L medium
Glycerol	100 g to 10 L medium
pH 7, adjusted with NaOH 1M	

The yeast-extract and glycerol solutions were mixed after sterilization.

B.2 Glycerol	50g to 10 L medium

2.2.4. Mass-culture Medium (Reisenberg *et al*.1990, modified)

Several fed-batch fermentation experiments were carried out using this medium. The feeding strategy followed the glycerol concentration in the fermenter medium (the feeding was performed when glycerol concentration was nearly zero).

A. Medium: Bas-HD1

	Solution concentration	Final conc. in bioreactor
1. Solution Bas-HD1		945 parts from 1000
1. KH_2PO_4	14.07 g/L	13.30 g/L
2. $(NH_4)_2HPO_4$	4.23 g/L	4.00 g/L
3. Citric acid monohydrate	1.80 g/L	1.70 g/L
4. SL-HD-B1 (see below)	1.06-g/L	1.00 g/L
2. Solution SL Thiamin		2 parts from 1000
Thiamin-hydrochloride (B1-hydrochloride vitamin)	4.50 g/L	9.00 g/L
3. Solution SL-$MgSO_4$		5 parts from 1000
$MgSO_4 \times 7H_2O$	216.02 g/L	1.80 g/L
4. Solution Glycerol		48 parts from 1000
Glycerol	630.00 g/L	30.24 g/L
5. Solution YE		
Yeast-extract	10.00 g/L	10.00 g/L

Solutions 1, 4 and 5 are separately sterilized by autoclaving and solutions 2 and 3 are sterile filtered using a 0,22 µm filter. All solutions are mixed together just before use.

Solution SL-HD-B1 (trace elements solution)

	Solution concentration
1. Fe (III)-citric acid monohydrate	100.80 g/L
2. $CoCl_2 \times 6H_2O$	2.50 g/L
3. $MnCl_2 \times 4H_2O$	15.00 g/L
4. $CuCl_2 \times 2H_2O$	1.50 g/L
5. Boric acid (H_3BO_3)	3.00 g/L
6. $Na_2Mo_5O_7 \times 2H_2O$	2.10 g/L
7. ZnAcetate $\times 2H_2O$	33.80 g/L
8. EDTA di-sodium $\times 2H_2O$	14.10 g/L

B. Feeding solutions

B.1 Yeast-extract 5 g/L
Glycerol 20 g/L

The ratio of 1:4 between yeast- extract and glycerol was changed for same experiments to 1:3 (fermentation in GBF laboratory).

B.2 Feeding solution HD1

	Solution concentration	Medium concentration
1. Solution Glycerol		961 parts from 1000
Glycerol	705.82 g/L	678.29 g/L
2. Solution SL $MgSO_4$ 400 g/L		38 parts from 1000
$MgSO_4 \times 7H_2O$	39.56 g/L	12.90 g/L
3. Solution SL-HD-F1		0.775 parts from 1000
1. Fe (III)-citric acid $\times H_2O$	40.00 g/L	31.00 g/L
2. $CoCl_2 \times 6H_2O$	4.00 g/L	3.10 g/L
3. $MnCl_2 \times 4H_2O$	23.50 g/L	18.21 g/L
4. $CuCl_2 \times 2H_2O$	2.30 g/L	1.78 g/L
5. Boric acid (H_3BO_3)	4.70 g/L	3.64 g/L
6. $Na_2Mo_5O_7 \times 2H_2O$	4.00 g/L	3.10 g/L
7. Zn acetate $\times 2H_2O$	16.00 g/L	12.40 g/L
8. EDTA di-soudium $\times 2H_2O$	13.00 g/L	10.07 g/L

To obtain the solutions SL-HD-B1 and SL-HD-F1, the substances had to be dissolved one by one and the solutions boiled and cooled down several times.

2.2.4. Mineral-Yeast-extract Medium

Fed-batch fermentation experiments were carried out using this medium. The feeding strategy followed the dissolved oxygen concentration in fermenter medium (the feeding was initiated after a defined period of time when oxygen saturation exceeded a pre-set value).

A. Medium

1. Yeast solution

1. Yeast-extract	40.00 g/L
2. $(NH_4)_2SO_4$	5.00 g/L
3. $Na_2HPO_4 \times 12H_2O$	7.75 g/L
4. $NaH_2PO_4 \times 2H_2O$	1.02 g/L
5. KCl	1.00 g/L
6. $FeSO_4 \times 1H_2O$	0.25 g/L
7. Citric acid	4.20 g/l

2. Solution $MgSO_4$

$MgSO_4 \times 7H_2O$	2.00 g/L

3. Thiamin Solution

Thiamin hydrochloride	5.00 mg/L

4. Trace elements solution

1. H_3BO_3	2.00 mg/L
2. $(NH_4)_6Mo_70_{24} \times 4H_2O$	0.80 mg/L
3. $CuSO_4 \times 5H_2O$	0.16 mg/l
4. KI	0.40 mg/L
5. $MnSO_4 \times H_2O$	2.02 mg/L
6. $ZnSO_4 \times 7H_2O$	1.60 mg/L

B. Feeding solution

Glycerol	600 g/L

3. Cultivation

3.1. Strain Storage

3.1.1. Strain Storage (-80°C)

A 12 hours old 50 ml LB medium+ ampicillin culture was centrifuged (Du Pont centrifuge, SS 34 rotor, 39,000 g, 10 minutes) under sterile conditions. Pellets were resuspended in 10 ml solution containing: 5 ml LB medium+ ampicillin
5 ml glycerol (p.a., 87%).
This suspension was divided in small portions (500 µl each) and stored in Kryo cups at –80°C after freezing in liquid nitrogen.

3.1.2. Strain Storage on Petri Plates

Both *E.coli* XL-1 blue/pMS*ift*OptR and *E.coli* XL-1 blue/pMS*ift*OptWT microorganisms are streaked on petri plates with fresh LB-Agar medium (see 2.1.2.), incubated over night at 37°C, and stored (protected against drying with Para film) at 4°C.

3.2. Cultivation in Shake Flasks

3.2.1. Preculture

One single colony from an agar plate (see 3.1.2.) was introduced into 10 ml glass tubes (13 mm diameter) containing 5 ml LB medium + 5µl ampicillin solution and covered with a metal cap. The cultivation proceeded for 16 hours in a shaker, at 37° and 170 rpm. Other incubation times than 16 hours are indicated.

3.2.2. Main Culture

A 5 ml preculture was used to inoculate the main culture. The ratio between the preculture and the main culture was of 1:100. A 2 chicanes flask with a volume of 5 times the volume of the culture was used. The incubation conditions are similar with the one of the preculture.

3.2.3. IPTG and Lactose Induction

IPTG solution	0.06 g/ml
Lactose solution	0.9 g/ml

A 500 ml main culture in a 2,5 L shake flask was cultivated as described above for 12 hours. For induction, 1 ml IPTG solution (end concentration 0.5 mM) was added and the incubation continued for another four hours.
The same procedure was followed for lactose induction. The lactose concentrations in the medium were 5 mM and 10 mM, when 1 ml was added, and 2 ml, respectively.

3.3. Cultivation in Bioreactors

3.3.1. Precultures

The first preculture for cultivation in bioreactors (so called fermentation experiments) was handled in the same way as a preculture for shake flasks (see 3.2.1.). The preculture medium and incubation time will be mentioned for every particular experiment.

The second preculture was similar with a main culture for shake flasks experiments (see 3.2.2.). The culture volume was 100 ml; the medium and the incubation time are specified below.

3.3.2. Main Culture

3.3.2.1. 1 L Bioreactor

In 1 L bioreactor (Biostat Q, Fa. Braun, Melsungen) fermentation experiments were performed with LB medium, yeast-extract-glycerol medium and mass culture medium.

Technical parameters for Biostat Q:

- stirring system: one disc with four pallets, ∅ = 28 mm
- maximum rotation speed: 1500 rpm
- pH electrode (Fa. Mettler Toledo, Switzerland) coupled with bioreactor pumps
- pO_2 electrode (Fa. Mettler Toledo, Switzerland)
- disposable filters for aeration and exhaust air (Fa. Braun, Melsungen)

A hose pump (Minipuls 2, Fa. Abimed, Langenfeld) was used to collect samples.

The maximum working volume for this bioreactor was 700 ml. The medium is sterilized in the bioreactor vessel in autoclave, and after cooling down the bioreactor is connected to the fermenter. The oxygen electrode is polarized for at least 6 hours and then calibrated with nitrogen and air. Only after the fermentation conditions were reached (37°C, 1500 rpm), the bioreactor was inoculated.
Sterile 1 M NaOH and 10% H_2SO_4 solutions were used to adjust the pH to 7 during fermentation.
A special function -so called cascade (that correlates the oxygen saturation in the bioreactor with the rotation speed) was used to maintain the value recorded by the oxygen electrode at 40%.

I. Fermentation with LB medium

To 700 ml sterilized LB medium, 0.1 ml antifoam solution (Ucolub 115, Fa, Brenntag) and 0.7 ml ampicillin were added with a sterile syringe. As inoculum, 7 ml from a second preculture (see 3.3.2.) were used.

II. Yeast extract-glycerol medium

For this medium, the starting volume was 600 ml, because 2 feeding steps (each time 50 ml) were taken into consideration. To 600 ml medium, 0.6 ml ampicillin solution were added. As antifoaming agent, 0.5 ml Struktol J 673 (Fa. Schill+Seilacher, Hamburg) were added. In order to maintain the inoculation ratio, 6 ml from a second preculture were introduced in the bioreactor.

To carry out the fed-batch strategy, solutions containing yeast-extract and glycerol were separately sterilized and injected into the bioreactor when glycerol concentration in the culture broth was almost zero. The glycerol concentration was measured by HPLC (see 11.3).

III. Mass culture medium

Starting volume was 650 ml, considering the fed-batch procedure. 614,25 ml solution BaS-HD1 (see 2.2.3.) were sterilized in the bioreactor. Under sterile conditions, 0.615 ml trace elements solution (SL-HD-B1), 1,3 ml thiamin solution, 3.25 ml $MgSO_4$ solution, 31.2 ml glycerol, 0.65 ml ampicillin and 0.5 ml antifoaming agent (Ucolub N 115, Brenntang, Oberhausen) were added. Respecting the inoculation ratio, the inoculation volume was 6.5 ml. 50 ml feeding solution HD1 (see 2.2.3 B.2) were injected into the bioreactor when the glycerol concentration was almost zero.

3.3.2.2. 20 l Bioreactor

In a 20 l bioreactor (Biostat E, Fa. Braun, Melsungen) fermentation experiments were performed with LB medium, yeast-extract medium, mass culture medium and mineral-yeast-extract medium.

Technical parameters for Biostat E:

- stirring system: three discs with six pallets each, ∅ = 89 mm
- maximum rotation speed: 1000 rpm
- 4 chicanes
- pH electrode (Fa. Ingold, Zürich, Switzerland) connected with a hose pump for base and acid dosage
- pO_2 electrode (Fa. Ingold, Zürich, Switzerland)
- disposable filters (Fa. Pall, Dreieich) for aeration and exhaust air
- external steam generator (Fa. Strubel, München) for sample collector sterilisation.

Samples were collected through a hose pump (Minipuls 2, Fa. Abimed, Langenfeld) coupled with a fraction collector device (Type 201 Controller, Fa. Abimed, Langerfeld).
For some experiments, a balance (Sartorius LA 12000 S, Germany) that monitors the consumption of the fed-batch solution was connected with the fermenter computer in order to follow a calculated feeding profile.

10 L medium (LB medium, yeast-extract-glycerol medium, mass-culture medium or mineral-yeast-extract medium, (see 2.2.) and 5 ml antifoaming agent were sterilized for 20 minutes at 121°C. After the fermentation temperature was reached (37°C), 10 ml ampicillin solution were added; 100 ml inoculum (second preculture) were used to start the experiment. The dissolved oxygen was kept at 20% or 40% of saturation (depending on the experiment) by a pO_2-agitation rate-aeration valve control loop.

I. Fermentations with LB medium, yeast-extract-glycerol medium and mass-culture medium are following the same steps as the ones for 1 L fermenter, but considering the higher volume, the inoculum and the feeding solutions are pumped in the fermenter. The volume of the fermenter allows a continuous fed-batch procedure for yeast-extract-glycerol medium and mass culture medium.

II. Mineral-yeast extract medium: 9900 ml yeast solution were sterilized in the bioreactor. After the fermentation temperature was reached, to the bioreactor were added 20 g $MgSO_4 \times 7H_2O$ dissolved in 50 ml dest water, 50 mg thiamin dissolved in 25 ml dest water, 25 ml trace elements solution (see 2.2.3.9) and 10 ml ampicillin solution. All these solutions were injected into the bioreactor with a 50 ml sterile syringe, and the inoculum (100 ml from a second preculture) was injected using a special inoculation syringe. The fed-batch solution was sterilized separately and introduced into the bioreactor through a hose pump.

4. Determination of Cultivation Parameters

4.1. Optical Density

The optical density (OD_{600}) was measured in 1 ml disposable plastic cuvettes at 600 nm using a Spectrophotometer (Shimadzu Spectrophotometer UV-120-02), against distilled water. For an optical density above 0,5 an appropriate dilution with distilled water was performed.

4.2. Wet Cell Weight (WCW)

SS 34 vials were weighed (M_1) and then filled with ca. 10 ml culture broth. After another weighing (M_2), vials were centrifuged for 10 minutes at 27,000 g in a precooled centrifuge (Sorvall, RC-5B Refrigerated Superspeed Centrifuge, Fa. DuPont Instruments, Bad Homburg, SS 34 rotor). The supernatant was removed and the tubes were turned upside down on a filter paper for 5 minutes. When all the remaining liquid was absorbed by the paper, the vials were weighed again (M_3). The wet cell weight ($g/kg_{culture\ broth}$ = $g/L_{culture\ broth}$) was calculated as follows:

$$WCW = \frac{(M3 - M1)}{(M2 - M1)} \times 1000 \ (g/L_{culture\ broth}) \qquad (9.1)$$

4.3. Dry Cell Weight (DCW)

After the wet cell weight was obtained, the tubes were dried over night at 60°C, cooled down in an exsiccator for one hour and then weighed (M_4). The dry cell weight value was obtained with the following formula:

$$DCW = \frac{(M4 - M1)}{(M2 - M1)} \times 1000 \ (g/L_{culture\ broth}) \qquad (9.2)$$

5. Determination of Protein Concentration

The protein concentration was determined following Bradford *(Bradford, 1976)*. This procedure is based on the Coomassie-Brilliant-Blue colorant binding to proteins. 20 µl sample were mixed with 1 ml Bradford reagent and incubated 5 minutes at room temperature. The extinction was measured at 584 nm using a Spectrophotometer (Shimadzu Spectrophotometer UV-120-02), against Bradford reagent + 20 µl dist. water. The calibration was made with BSA (Bovine Serum Albumin).

Bradford Reagent:

Serva Blau G-250	70 mg
Ethanol 96% (v/v)	50 ml
H_3PO_4 85% (v/v)	100 ml
H_2O dist.	to 1000 ml

The colorant was dissolved in ethanol, phosphoric acid was added and then filled up with distilled water to 1000 ml. The reagent was filtered and kept at 4°C protected from light.

6. Cell Disruption

During the production stage, the *E.coli* cells accumulate Inulase II intracelulary without any possibility to excrete it. In order to release the enzyme, the cells have to be destroyed and this procedure was performed using different mechanical methods.

6.1. Mixer-mill

Bacterial cells were harvested by centrifugation in a refrigerated centrifuge and suspended in buffer (0.04 M Sörensen phosphate buffer containing 2mM Mg^{2+}, pH 7, see below) to obtain a 2% WCW cell suspension. 4 g of this suspension, and 0.8 µl Benzonase (Merck KGaA, Darmstadt, Germany) (0.2 µl per g of wet cells) were placed in each stainless grinding chamber (chamber volume 30 ml). 8 g glass beads were added (diameter 0.3 mm) (ratio 1:2 between wet cell suspension and glass beads). After 30 minutes treatment in the mixer-mill (Retsch MM 2000, Windaus Labortechnik, Clausthal-Zellerfeld, Germany) at 100% intensity, the chambers were emptied using a spatula and washed with 4 ml buffer (0.04 M Sörensen phosphate buffer, pH 7). Both disruption mixture and washing buffer were collected in the same tube (SS 34 vials), which was further centrifuged to remove the cell debris for 10 minutes at 27,000 g in the refrigerated centrifuge. The clear supernatant (approximately 8 ml) was used for enzyme analysis. The glass beads were repeatedly used after sterilization, washing and drying at 50°C over night.

Sörensen phosphate buffer + Mg^{2+} (0.04 M, pH 7)

Na_2HPO_4	4.360 g/L
KH_2PO_4	2.110 g/L
$MgSO_4 \times 7H_2O$	0.493 g/L

6.2. Ultrasonication

Unless otherwise stated, the following standard procedure was used for cell disruption. 0,5 ml from the culture broth were centrifuged in a 10 ml glass test tube (∅=13mm) using a Jouan Centrifuge (BR.4, Jouan Centrifuge, France) (rotor S40, 2,100 g, 10°C for 10 minutes). After centrifugation, the supernatant

was removed by inverting the test tubes until all medium had been drained. Pellets were suspended in 5 ml of cold NaCl 0,9% (from ice) using a vortex device (dilution 1:10 regarding the native culture broth).

For samples with OD_{600} > 20, this pellet suspension was diluted one more time with cold NaCl 0,9% (0.5 ml pellet suspension + 4.5 ml NaOH solution, dilution 1:100 compared to native culture broth).

The cell suspension was then assayed for disruption, using an ultrasounds device (Sonopuls Homogeniser, Bandelin *electronic* GmbH & CoKG, Berlin, Germany) with the following parameters:

- probe: KE76
- cycle: 5×10%
- power: 60%
- time: 2 minutes

The probe was not more than 2 cm immersed in the cell suspension, with no contact to the walls of the test tube. To prevent overheating, the test tubes were placed on ice during cell disruption. At the end, 2 ml from the disrupted cell suspension were further centrifuged in 2 ml Eppendorf cups for 5 minutes (Jouan centrifuge, rotor AB 2.14, 9,400 g, 4°C) and the supernatant was assayed for enzyme analysis.

6.3. High-Pressure Homogenizer

A cell suspension containing 100 g cells in 1 L 0.04 M Sörensen phosphate buffer, pH 7 (10% WCW) was assayed for cell disruption in a high-pressure homogenizer. To achieve an efficient disruption, the cell suspension was pumped two times through the homogeniser, with the following working parameters:

- time: 10 minutes
- pressure: 650 bars

500 ml phosphate buffer were used to remove the cells from the device, and so the pellet suspension was diluted regarding the initial cell suspension assayed for disruption. The temperature was measured before and after disruption.
The cells debris was removed using a refrigerated centrifuge (Du Pont centrifuge, rotor GS3, 11,000 g, for 10 minutes). The supernatant was stored at –20°C and used for further enzyme analysis.

The homogenisation was kindly carried out by the GBF.

7. Plasmid DNA Investigations

The plasmid DNA investigations were made to check the presence of the plasmid (43 kb) in the E. *coli* cells. The plasmid digestion was meant to investigate the integrity of the plasmid which consists of the pUC 19 vector (2.7 kb) and a 1.7 kb insert (coding for Inulase II). Both fragments should be visible in gel electrophoresis. A single digest was carried out to check for plasmid size (remove super-coiled plasmid DNA).

7.1. General precautions

All devices and solutions were autoclaved 20 minutes at 121°C to inactivate nucleases. The devices that could not be sterilized were washed with 70% ethanol. Temperature sensitive solutions were sterile filtered (0.22µm, Millex-GS).
The plasmid DNA isolation was performed using a Qiagen® Plasmid Midi Kit (Qiagen GmbH, Hilden, Germany). The kit contained ready-to-use buffer solutions and the isolation procedure was performed following the Qiagen® Plasmid Purification Handbook.

7.2. Plasmid DNA isolation

A culture of 25 ml LB medium including ampicillin was centrifuged under sterile conditions at 39,000 g, 4°C for 15 minutes (Sorvall centrifuge, SS 34 rotor). The bacterial pellets were resuspended in 4 ml Buffer P1 previously supplemented with RN-ase A. After the complete resupension using a vortex device, 4 ml of Buffer P2 were added and gently mixed by inverting the vial 4-6 times and then incubated at room temperature for 5 minutes. Next, 4 ml of chilled Buffer P3 were added and the sample was incubated on ice for 15 minutes. The lysate was cleared by filtration using a QIAfilter Midi Cartridge and plasmid DNA bound to the QIAGEN resin from an QIAGEN-tip equilibrated with 4 ml of Buffer QBT. The QIAGEN-tip was washed 2 times with 10 ml Buffer QC and the DNA was eluted with 5 ml Buffer QF. 3.5 ml isopropanol were added to precipitate the eluted DNA and the sample was centrifuged at 18,000 g, 4°C for 30 minutes (Jouan Centrifuge, AB 2. 14. rotor). The DNA pellets were washed with 2 ml of room temperature 70% ethanol, centrifuged again at 18,000 g for 10 minutes and redissolved in Buffer TE. They were stored at 4°C.

7.3. Digest of plasmid

The plasmid DNA was further assayed for cleavage using the restriction endonucleases Hind III and EcoR I (QBIOgene, Heidelberg).
First cleavage was performed with Hind III enzyme prepared as follows:

- 5 µl enzyme Hind III
- 36 µl Buffer II (see below)
- 283 µl bidist. H_2O

were mixed carefully pipetting up and down.

27 µl enzyme solution and 3 µl plasmid DNA (7.2.) were mixed and incubated at 37°C for 3 hours. The enzymatic reaction was stopped placing the sample in a 68°C water bath for 10 minutes.

The second cleavage was made with EcoR I enzyme: 0.3 µl 5M NaCl and 0.4 µl enzyme EcoR I were added and the sample was incubated at 37°C for 3 hours. The enzyme was inactivated heating the sample at 68°C for 10 min.

The enzymatic digest of plasmid DNA was performed with enzymes and buffer solutions from Fa. QBIOgene, Heidelberg, Germany.

Buffer II composition (pH 8):

	Final concentration (mM)
Tris·HCl	10
NaCl	50
$MgCl_2$	10
2-mercaptoehanol	10
BSA(in mg/ml)	0.1

7.4. DNA-Agarose Electrophoresis

The single and double digest plasmid preparation were assayed for horizontal agarose electrophoresis in a 10.0 × 6.5 cm running chamber (Fa. Schütt labortechnik GmbH, Gottingen). To prepare the 0.7% agrose-gel, 2.1 g agarose were solubilized in 300 ml TAE buffer (see below), also used as running buffer, by boiling the mixture for 1 minute in a microwave oven and then 6 µl ethidiumbromide solution (from a 5 g/L solution) were added. 30 µl plasmid digest and 2 µl Stop-Mix solution (see below) were mixed and 28 µl from this mixture were pipetted in every well. This solution contains bromophenolblue and so it is possible to follow the running line. The standard Felix TM-500 bp DNA Ladder (Fa. QBIOgene) was used to determine the sample DNA size. The electrophoresis was carried out at 80 V and was finished when the running line reached the end of the gel (after about 1 –1 ½ hours).

50 × TAE-Buffer (Sambrook *et.al*., 1989)

TRIS	242 g	2 M
EDTA (0.5 M; pH 8.0)	100 ml	50 mM
acetic acid (conc.)	57 ml	1 M
H_2O bidest	to 1000 ml	
pH 8.5		

Stop- Mix (Sambrook *et.al*., 1989)

EDTA	19 mg	1 mM
urea	21g	7 M
saccharose	25 g	50% (w/v)
brompfenolblue	50 mg	0.1% (w/v)
H_2O bidest	to 50 ml	
pH 7.0		

7.5. Staining with ethidiumbromide

The ethidiumbromide intercalates in the DNA helix and due to its fluorescence the DNA bands can be visualized using an UV-Transilluminator (λ=254 nm) placed in a dark-room (Pixel Room, Fa. Schütt labortechnik, Göttingen) (for camera and software see 8.2.).

8. Protein Electrophoresis

8.1. Procedure of gel electrophoresis

The protein electrophoresis was performed using 12.5% SDS precast gels (Anamed Elektrophorese GmbH, Darmstadt, Germany), allowing the loading of 12 samples a 25 µl each. The running chamber and the power pack (Standard Power Pack P25) were supplied by Biometra biomedizinische Analytik GmbH, Göttingen, Germany, and the standard (Precision Protein Standard) by Bio-Rad

Laboratories GmbH, München). First, the samples had to be denatured by dilution with sample buffer and heating at 100°C, 600 rpm for 10 min (Eppendorf Thermomixer Comfort, Fa. Windaus Labortechnik, Clausthal-Zellerfeld, Germany). 2 types of protein buffer, handled as follows, were used:

	Dithiothreitol (DTT) (mg/ml buffer)	Ratio sample: buffer
• Laemmli Sample Buffer (Bio-Rad Laboratories)	54	2:1
• Anamed Sample Buffer (Anamed Electrophorese GmbH)	54	1:1

The treatment of the buffer with reduction agent (DTT) was made just before use. After denaturation, samples were centrifuged at 8,500 g for 3 minutes (Eppendorf Centrifuge 5415C). The sample wells were loaded with 5µl for the protein standard and 25 µl for samples using a Hamilton syringe. The power supply was fixed at 10 mA for 10 minutes and then at 25 mA for the rest of running time. Electrophoresis was stopped when the running line was nearly at the edge of the gel.

Electrode buffer:

TRIS	2,9 g/L
Glycine	14.4 g/L
SDS	1.0 g/L

pH ~ 8 (not adjusted with acid or base)

8.2. Staining

8.2.1. Coomassie Brilliant Blue Staining

The unspecific protein staining was performed according to Weber and Osborn (1969).

I. Reagents:

Fixing solution	trichloroacetic acid	200 g
	H_2O_{bidist}	to 1000 ml
Staining solution A	Coomassie Brilliant Blue R 250	0,2 g
	methanol	to 100 ml
Staining solution B	acetic acid	200 ml
	H_2O_{bidist}	to 1000 ml
Destaining solution	acetic acid	100 ml
	methanol	200 ml
	H_2O_{bidist}	to 1000 ml

II Staining procedure:

Fixation	30 minutes
Mixed staining solution (solution A: solution B = 1:1 (v/v))	60 minutes
Destaining solution	until the background is decolourised

The solutions A and B were mixed just before use; the destaining solution had to be changed several times until the background was properly decolourised. The stained gels were stored in plastic folia with small amounts of water at 4°C.

8.2.2. Silver staining

The silver staining procedure is a very sensitive one, which allows an intensive staining of small protein concentrations and minimal background *(Poehling and Neuhoff, 1981).*

I. Reagents:

Fixing solution	ethanol	500 ml
	acetic acid	120 ml
	H_2O_{bidist}	to 1000 ml
Ethanol solution	ethanol	500 ml
	H_2O_{bidist}	to 1000 ml
Thiosulphate solution	$Na_2S_2O_3 \times 5H_2O$	40 mg
	H_2O_{bidist}	to 200 ml
Staining solution	$AgNO_3$	0.2 g
	37% formaldehyde	75 µl
	H_2O_{bidist}	to 100 ml
Development solution	Na_2CO_3	12 g
	thiosulphate solution	4 ml
	37% formaldehyde	0.1 ml
	H_2O_{bidist}	to 200 ml
Stop solution	EDTA	18.6 g
	H_2O_{bidist}	to 1000 ml

II. Staining procedure:

Fixing	1-24 hours
Ethanol solution	3 × 1 minute
Thiosulphate solution	1 minute
H_2O_{bidist}	3× 20 seconds
Staining	15-25 minutes
H_2O_{bidist}	10 seconds
Development	until the bands were visible
Stop solution	5 minutes
H_2O_{bidist}	1 hour

The thiosulphate, staining and development solutions were freshly prepared for every experiment. The other solutions could be kept for several weeks at 4°C.

The gels were placed in a dark room (Pixel Room, Fa. Schütt Labortechnik, Göttingen) connected with a digital camera (Nikon Coolpix 450). The pictures were analysed with Gelscan computer software.
The stained gels were stored in plastic folia with small amounts of water at 4°C.

9. Immobilization

9.1. General Immobilization Procedure

For a repeated use and improved stability, enzymes can be immobilized to diverse carrier materials. For this work, an immobilization to anion exchange resins was investigated.

9.1.1. Resin equilibration

The following resins were employed (Fa. Rohm and Haas Deutschland GmbH, Frankfurt/ Main):

Duolite A561
Duolite A568
Amberlite 94S
Amberlite IRA 67

100 g resin and 1 L phosphate buffer (pH 9) were mixed and shaken (Edmund Bühler Swip KS 10) at room temperature for at least 4 hours (ratio resin: buffer = 1:10). The equilibrated resin was washed several times and could be stored for a long time in small amounts of distilled water.

Phosphate buffer:

Na_2HPO_4	35.6g/L
KH_2PO_4	27.2g/L

The phosphate solutions were separately prepared and mixed in equal volumes. The pH of the obtained solution was around 6.7 and was adjusted to 9 with 10% NaOH.

9.1.2. Enzyme immobilization

5 g equilibrated resin were mixed and shaken with 50 ml enzyme solution (ratio resin: enzyme = 1:10) for 24 hours at room temperature. In order to investigate the amount of unbound enzyme after immobilization, 1 ml from the supernatant was kept for further investigations. The supernatant was then decantated and the resin with immobilized enzyme was washed several times with dist. water.

9.1.3. Cross-linking with Glutardialdehyde

The immobilized enzyme was shaken in a ratio 1:5 with 0.3% glutardialdehyde for 3 hours at room temperature. The supernatant was removed and the resin with immobilized enzyme was washed 4-5 times with dest. water. The immobilized enzyme was further assayed for enzyme analysis or stored in small amounts of water at 4°C.

9.2. Adsorption

For this experiment, enzyme solutions with different activities were immobilized. The activities were between 230,000 and 1,000 U/L, and were obtained diluting the initial enzyme solution with an appropriate amount of distilled water. The

resin equilibration, enzyme immobilization, and cross-linking procedures were as described above.

9.3. Repeated Biotransformation of Inulin

The immobilized enzyme was assayed for repeated biotransformation of Cosucra inulin. The immobilization was performed following the standard procedure.
6 g of *E.coli/*pMS*ift*OptR immobilized enzyme with an initial activity of 60,000 U/L were used for this experiment and so the enzyme stock solution, with an activity of 1.500.000 U/L had to be 1:25 diluted with dist water.

10. Enzyme Analysis

Inulase II degrades inulin to DFAIII and FOS (GF_2, GF_3, GF_4). The enzymatic activity of the enzyme is determined from the amount of DFAIII produced. 1U of enzyme responds to 1μmol DFAIII per minute. A standard assay for enzyme activity was carried out as follows.

Inulin solution

100 g inulin/L were dissolved in 0.04 M phosphate buffer pH 5.25 by heating the solution at 90-100°C under stirring. When the inulin was completely dissolved the solution was cooled down to 50-60°C using an ice bath. This solution was freshly prepared before use and will be further referred to as Inulin solution.

Two different sorts of inulin were employed: Dahlia inulin with a polymerization degree ~30 and Cosucra inulin with DP~10.

Cosucra inulin contains small amounts of DFAIII, which had to be determined for every IPG solution and so a blank sample was prepared (see 10.1.1.).

Phosphate buffer 0.04 M

$Na_2HPO_4 \times 2H_2O$	0.14 g/L
KH_2PO_4	5.33 g/L
pH 5.25	

10.1 Intracellular Inulase II

10.1.1. Standard Enzyme Assay for Fermentation Experiments

100 µl enzyme solution and 900 µl Inulin solution (100 g/L) were incubated in 2 ml Eppendorf cups at 50°C for 30 minutes. The enzymatic reaction was stopped boiling the samples at 100°C for 5 minutes. 10 µl Novozym SP 230 were added (to hydrolyze the remaining inulin to fructose and glucose) and samples were incubated at 60°C for another 30 minutes. For desalting, ca. 150 mg ion-exchanger were introduced in every cup, and then were shaken at room temperature for 30 minutes (Edmund Buhler Swipe KS10). Samples were filtered through a 0.22 µl filter and measured by HPLC.

The enzyme solutions had to be diluted with water for a final concentration of DFAIII in the reaction tube of around 3 g/L.

The blank sample to determine the DFAIII from Cosucra inulin was prepared in a similar way as a normal sample, but instead of enzyme solution, 100 µl H_2O were used.

10.1.2. Standard Enzyme Assay to Determine the Kinetic Parameters

The Michaelis-Menten kinetic parameters were determined performing activity tests as described above, but the enzymatic reaction was stopped after 5, 10, 15, 20, 30 minutes and the inulin concentration in IPG solution was varying between 3 g/L and 120 g/L.

10.1.3. Assays for Immobilized Enzyme

2 g immobilized enzyme and 20 ml IPG were shaken at 150 rpm for 30 minutes in a 50°C water bath. 1 ml liquid sample (without resin) was collected after 5, 10, 20 and 30 minutes and heated at 100°C for 5 minutes. Then, the samples were handled as described above (see 9.1.1.). To determine the Michaelis-Menten kinetic parameters of immobilized enzyme, the substrate concentration was varied between 3 and 120 g/L, as for the free enzyme.

10.1.4. Repeated Biotransformation of Inulin

6 g of immobilized enzyme were introduced into a separation funnel placed into a 60°C oven (Heraeus T 5024, Hanau, Germany). 1,53 L phosphate buffer pH 5.25 containing 250 g Cosucra inulin was pumped in circle by a hose pump through the funnel. To prevent the inulin from falling out, the solution was maintained at 80°C using a hot water bath. Between the inulin reservoir and funnel a 60°C water bath was placed to make sure that the inulin solution that reaches the immobilized enzyme has the proper temperature for the enzymatic reaction and does not inactivate the enzyme by temperature $>$ 60°C. Every 24 hours the Inulin solution was replaced with a new one. The DFAIII concentration obtained after 24 hours was determined from 1 ml inulin solution and performing the same

steps as for a general activity test (hydrolyses with Novozym and desalting with ion-exchanger).

10.3. Determination of Inulase II in Culture Supernatant

Following the fermentation experiments, the culture broth was centrifuged and the supernatant was assayed for activity test in order to investigate the presence of Inulase II in the culture medium due to cell lyses. A standard activity test was performed (see 9.1.1.), using 100 µl supernatant instead of 100 µl enzyme solution.

11. HPLC Analysis

High-pressure liquid chromatography (HPLC) was the analytical method to determine the concentration of DFAIII in enzymatic assays and to follow glycerol concentration in fermentation experiments.

11.1. Equipment and running parameters

Degasser	Online Vakuumentgaser 3322, Fa. ERC, Alteglofsheim
Pump	HPLC Pump 64.00, Titan, Fa. Dr. H. Knauer, Berlin
Detector	RI-Detektor ER-7512, Fa. ERC, Alteglofsheim
Oven	Mistal- Column oven, Fa. Spark Holland, Holland
Auto sample	Marathon-Auto sampler, Fa. Spark Holland, Holland
Integrator	Chromeleon Software
Ca^{2+} Column	Aminex HPX-87 C, 300 × 7.8 mm, Fa. Bio-Rad Laboratories, Richmond USA

Precolumn	Carbo-C Refill Cartridges, 30 × 4.6 mm, Fa. Bio-Rad Laboratories, Richmond USA
H^+ Column	Aminex Fermentation Monitoring Column, 150 × 7.8 mm Fa. Bio-Rad Laboratories, Richmond USA

Temperature	80°C
Mobile phase	Bidest. H_2O
Pressure	$3.1 \times 10^6 – 4.6 \times 10^6$ Pa
Flow rate	0.7 ml/minute
Injection volume	20 µl

11.2. DFAIII

On a Ca^{2+} column (Aminex HPX-87 C), along with DFAIII, glucose and fructose could be separated and quantified. The calibration was performed using an external standard containing 4 g/L DFAIII (±0.001 g/L), intercalated between samples (one standard every 2 samples). The DFAIII concentration was calculated by peaks integration using Chromelon Software. The retention times for every component were:

	Retention time (minutes)
DFAIII	~7
Glucose	~8
Fructose	~11

The analysis time was 15 minutes for every sample.

The enzyme activity was calculated considering that 1U enzyme responds to 1µmol DFAIII per minute under standard conditions according to the formula 9.3.

$$\frac{\mathrm{U}}{\mathrm{L}}\left[\frac{\mu\mathrm{mol}}{\mathrm{L}\times\mathrm{min}}\right]=\frac{\mathrm{c(DFAIII)}\left[{}^{\mathrm{g}}\!/\!_{\mathrm{L}}\right]\times Vassay\ \left[\mathrm{ml}\right]}{\mathrm{MwDFAIII}\left[{}^{\mathrm{g}}\!/\!_{\mathrm{mol}}\right]\times time\left[\mathrm{min}\right]\times V\mathrm{enzyme\,solution}\left[\mathrm{ml}\right]}\times 10^6 \qquad (9.3)$$

Where:- c(DFAIII): DFAIII concentration measured by HPLC, multiplied with the HPLC dilution

- V_{assay}: volume of enzyme + Inulin solution
- M_w(DFAIII): molecular weight of DFAIII (324 g/mol)
- $V_{enzyme\ solution}$: the volume of enzyme assayed for activity test

11.3. Glycerol and acetate

To adjust the feeding profile in fermentation experiments, a rapid determination of glycerol and acetate concentrations was necessary.

Glycerol and acetate concentrations were also measured using the HPLC with a H^+ column (Aminex Fermentation Monitoring Column). The calibration was made with an external standard containing 5 g/L glycerol and 2.5 g/L acetate. The retention times were:

	Retention time (minutes)
Glycerol	~4
Acetate	~5

The peaks integration was made with Chromelon Software and one HPLC run took 10 minutes.

2 ml culture broth from a fermentation experiment were centrifuged at 8,500 g for 3 minutes (Eppendorf Centrifuge 5415C, Fa. Wilh. O Schmidt GmbH, Braunschweig). The supernatant was directly assayed for HPLC analysis

12. Substances

Beside the substances from Merck, Darmstadt (with analytical purity) and Riedel-de Haen, the following substances were used:

Substance	Producer
Inulin	Cosucra, Momalle, Belgium
Novozym 230	Novo Nordisk, Mainz
Ucolub N 115	Brenntag, Oberhausen
Yeast extract	Merck, Darmstadt
Peptone from casein	Merck, Darmstadt
Serva Blue G	Serva, Heidelberg
Ampicillin	Roth
Duolite A561	Rohm and Haas Deutschland GmbH, Frankfurt/ Main
Duolite A568	Rohm and Haas Deutschland GmbH, Frankfurt/ Main
Amberlite IRA 67	Rohm and Haas Deutschland GmbH , Frankfurt/ Main
Amberlite 94 S	Rohm and Haas Deutschland GmbH , Frankfurt/ Main
Tris Base	Sigma Chemical Company, USA
SDS	Aldrich
Hind III	Q.BIOgene, Heidelberg,
EcoR I	Q.BIOgene, Heidelberg
Benzonase	Merck Darmstadt,
Dahlia Inulin	Serva, Heidelberg

Chapter 9

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Symbols

c	concentration
DCW	dry cell weight
DFA III	di-D-fructofuranose 1,2´:2,3´ dianhydride
DNA	deoxyribonucleic acid
DP	polymerization degree
DTT	dithiothreitol
E	enzyme
EDTA	ethylenediaminetetraacetate
ES	enzyme - substrate complex
FOS	fructo-oligosaccharides
GF_2	kestose
GF_3	nystose
GF_4	fructosyl-nystose
HCDF	high-cell density fermentation
HPLC	high-performance liquid
IPTG	isopropyl-β-D thiogalactopyranoside
kb	10^3 bases
kDa	10^3 Dalton
K_M	Michaelis-Menten constant
OD	optical density
P	product
rpm	rotations per minute

S	substrate
$[S]$	substrate concentration
SDS	sodium dodecyl sulfate
U	Unit= µmol/min
v	reaction rate
$v_{\max}$	maximal reaction rate
t	time
WCW	wet cell weight
$[\alpha]_D^{20}$	optical rotation
µ	specific growth rate
$\tau_{1/2}$	catalytic half-life